UNIVERSITY OF LONDON THESIS

Degree PhD Year 2005 Name of Author MARSH S.

Thesis presented by Sally Marsh
for the degree of Doctor of Philosophy
at the University of London.

Department of Biology
Darwin Building
Gower Street
London
WC1E 6BT

September 2004

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ABSTRACT

The impact of traffic pollution on the nitrogen metabolism and zinc content of roadside vegetation was investigated by measuring a range of physiological responses and surface Zn in trees.

Dry deposition in the form of NO_x ranged from 14.8 to 40.7 Kg N ha yr^{-1} within the woodland. The amount of deposition was spatially related to the distance from the motorway that runs adjacent to the woodland. Maximum NO_x deposition was found next to the motorway in the rural downwind environment. At this site trees exhibited significantly higher growth rates as assessed by yearly shoot extension as well as exhibiting more positive foliar $\delta^{15}N$ signatures compared to trees at a control site 600m away. These two results are taken as an indication that atmospheric NO_x produced by motorway traffic is contributing to the nutrition and growth of roadside trees.

In vivo activity of the N assimilatory enzyme, NR was assayed in the foliage of *S. nigra*, *C. monogyna*, *B. pendula* and *Quercus spp.* growing at the woodland. Elevated NR activity close to the road was only demonstrated for one species, *S. nigra* suggesting the assimilation of traffic-derived NO_x following foliar uptake. High NR activities in this species may mean that leaves act as NO_x sinks, effectively 'mopping up' NO_x from the atmosphere.

In general, proximity to the traffic appeared to have few effects of the motorway on tissue metabolite pools. Total N and tissue NO_3^- were not affected by proximity to the motorway. Similarly, no significant differences were found for total P concentrations. It is suggested that at this lowland deciduous woodland, soil nutrients are non-limiting and thus allow growth to occur in response to inputs from traffic-derived N. It is proposed that this increase in biomass has the effect of diluting elements or metabolites such as total N and NO_3^- so that they remain at a constant level. It is recommended that at similar sites, rather than using metabolites as biomarkers, foliar $\delta^{15}N$ signatures in combination with simple growth measurements are used as to demonstrate traffic effects.

Whereas results indicated that tissue Zn was unaffected by proximity to the road, surface Zn was found to accumulate during dry weather, only to be washed off during rainfall events. Amounts of surface Zn were examined on a 5 tree species growing in central London, namely *S. nigra*, *C. monogyna*, *F. excelsior*, *A. hippocastanum* and *P.acerifolia*. Surface Zn was highest on the leaves of *C. monogyna* which contained 13.8 mg Zn m^{-2} on its leaves. Scanning electron microscopy was able to show that species differences may be related to epicuticular wax characteristics. Results are discussed in relation to the potential use of trees for ameliorating atmospheric NO_x and particulate pollution levels.

This thesis is dedicated to my astonishingly kind friend,

Jonathan Lee.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ANCA-MS	Automated $^{15}N/^{13}C$ analysis – mass spectrometry
EDS	Energy dispersive microanalysis
GOGAT	Glutamate synthase
GS	Glutamine synthetase
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate) reduced
NO	Nitrogen monoxide
NO_2	Nitrogen dioxide
NO_3^-	Nitrate
NO_2^-	Nitrite
NO_x	Nitrogen oxides
NH_3	Ammonia
NH_4^+	Ammonium
NiR	Nitrite Reductase
NH_y	Ammonium and Ammonia
NR	Nitrate Reductase
O_3	Ozone
PM	Particulate matter
$PM_{2.5}$	Particulate matter of diameter smaller than 2.5 μm
PM_{10}	Particulate matter of diameter smaller than 10 μm
SEM	Scanning electron microscope
SOM	Soil organic matter
VON	Volatile organic nitrogen
Zn	Zinc

ACKNOWLEDGEMENTS

I would like to give extra special thanks to the following people –

John Pearson

Tony Miller

Xiaohong Xhang

However I would also like to thank –

My parents

Everyone at Rainbow

William Purvis and Jean Devonshire for assistance and advice with SEM work.

My Friends – in particular those who have distracted me the most, namely Diana,

Pauline, Catherine, Carl and Jon.

GANE for funding this work

No thanks whatsoever are given to –

The spider mites

Stephen Thompson

My Appendix

Autumn

CHAPTER 1

INTRODUCTION

1.1. History of Air Pollution

Air pollution in the UK is not a recent problem. Pollution in the UK dates back to medieval times when coal combustion and other industries emitted heavy smoke to London skies (Bell and Treshow, 2002). In 1661, the first record of damage to vegetation as the direct result of aerial pollution was made by a British author and founding member of the British Royal Society, John Evelyn. In his pamphlet entitled *Fumifugium*, Evelyn describes thick dark smog over London. Such smogs were the result of the incomplete combustion of fossil fuels by industry. They contained carbon monoxide (CO), organic compounds and sulphur dioxide (SO_2). Over time, reactions with oxygen cause organic and sulphuric acid to form in droplets which gave the smog a haze-like appearance. As well as recommending the relocation of industries to the city outskirts Evelyn proposed the planting of orchards and gardens as a way of reducing pollution effects in the city. Despite his suggestions pollution in London has not decreased significantly until relatively recent years. In 1952 the Great London Smog caused around 4,000 deaths in the city and lead to the introduction of the Clean Air Acts of 1956 and 1968.

The decline in heavy industry and a switch from coal-generated to gas-powered electricity has brought with it a decline in SO_2 emissions in the UK. Emissions have decreased dramatically from around 6.5 million tonnes per year in 1970 to around 1 million tonne per year in 2004 (Figure 1.1). As a result of the decrease in SO_2 emissions, UK farmers now need to add sulphate fertiliser to some crops (Zhao *et al.*, 1999a, 1999b).

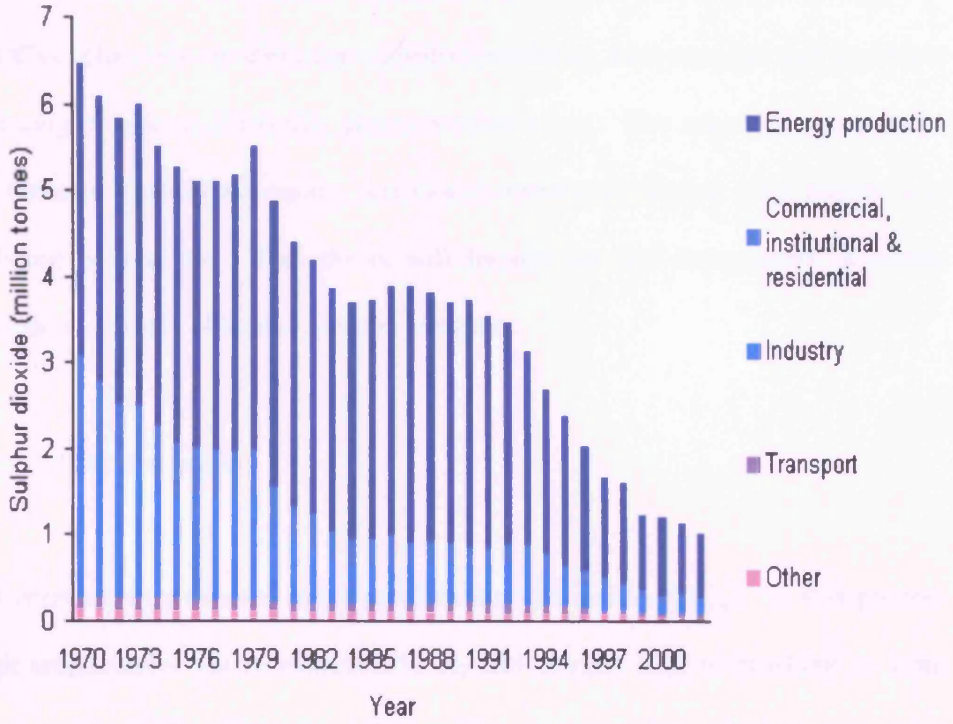


Figure 1.1. Sulphur dioxide emissions and sources in the UK since 1970. Graph obtained from National Atmospheric Emissions Inventory (NAEI) at www.aeat.com

1.2. Contribution of traffic emissions to atmospheric pollution

While atmospheric SO_2 has declined in recent years, the increased use of the motor vehicle has brought with it a range of other harmful pollutants. In the early 1980's the main interest was the effects of lead pollution but by the late 1980s and early 1990s the effects of other vehicle pollutants became a major concern. Traffic emissions contain a cocktail of pollutants including heavy metal particulates, gaseous nitrogen oxides (NO_x) and volatile organic compounds (VOCs). Not only are these components harmful on their own but they can react in sunlight to form damaging photochemical smog. The major components of photochemical smog are ozone, peroxyacyl nitrates, aldehydes, alkyl nitrates and airborne particulates. This thesis will focuses on two components of traffic emissions, namely NO_x and particulate matter.

1.2.1. Nitrogen oxides

All combustion processes in air produce nitrogen oxides (NO_x). In this process high temperatures cause atmospheric N_2 and oxygen (O_2) to combine to from nitric oxide (NO) (Welburn, 1994). The reaction takes place in two steps (Reactions 1.1 and 1.2). NO can also be formed in fuel rich regions of the flame from highly reactive hydroxyl radicals ($^{\cdot}OH$) (Reaction 1.3).

$$N_2 + O \rightarrow NO + N \quad (1.1)$$

$$N + O_2 \rightarrow NO + O \quad (1.2)$$

$$N + {}^{\cdot}OH \rightarrow NO + H \quad (1.3)$$

Nitrogen dioxide (NO_2) is formed from the oxidation of NO by O_3 (Reaction 1.4).

$$O_3 + NO \rightarrow NO_2 + O_2 \quad (1.4)$$

A range of subsequent reactions may remove NO_2 from the atmosphere and the amount of NO_2 and NO in the air will be dependent on the rate of these reactions. In remote unpolluted areas, NO concentrations are generally low compared to NO_2 concentrations. However NO concentrations may exceed those of NO_2 in heavily polluted towns and cities where the oxidising capacity of the atmosphere may be limited (The Air Quality Strategy, 1999). Road transport accounts for about half of national annual emissions of NO_x. The road transport contribution is fairly evenly split between petrol (26%) and diesel emissions (22%) (The Air Quality Strategy, 1999). Concentrations of NO_2 and oxides of nitrogen are currently monitored using automatic instruments at 83 national network sites across the UK. The emissions of NO_2 in the UK are shown in Figure 1.2. Concentrations are highest around large cities and along major roads.

1.2.2. Production of atmospheric NO_x from a major road in a rural environment

In the document, *Nitrogen Dioxide in the United Kingdom* (Air Quality Expert Group, 2004) the production of atmospheric NO_x by traffic on a major road is described. Motorway traffic acts as a large source of NO_x, mainly as NO and with a small percentage of emissions as NO_2.

Figure 1.2. Emission map for nitrogen oxides as NO_2 in 2001.

From National Atmospheric Emissions Inventory

(www.naei.org.uk)

As air passes over the motorway it initially has pollutant levels typical of a relatively unpolluted rural environment. However, ozone (O_3) in the air reacts with the high concentrations of NO at the motorway to form NO_2 and O_2. This reaction occurs within a few seconds, corresponding to a spatial scale of up to a few tens of meters. As a result, both primary and secondary NO_2 concentrations reach a maximum in the rural downwind environment. Vertical dispersion acts to reduce secondary NO_2 concentrations, which decline rapidly with increasing height and distance from the motorway (The Air Quality Strategy, 1999).

1.2.3. Particulate Matter

Particulate matter (PM) is a complex mixture of organic and inorganic substances present in the atmosphere as both liquids and solids (Quarg, 1993). Particulate matter is a major factor in respiratory illnesses with more deaths occurring annually as the result of traffic emissions than due to traffic accidents (WHO, 1999). It is standard for particulate matter to be defined by its size rather than its chemical composition. Particles with aerodynamic diameters of 10 μm and 2.5 μm are known as PM_{10} and $PM_{2.5}$ respectively.

According to the 1999 APEG report the major sources of airborne PM_{10} in most urban areas are:

1. road traffic emissions (may contain heavy metals such as lead or zinc)
2. secondary particles (formed in the atmosphere from chemical reactions)
3. resuspension of soils and road dust

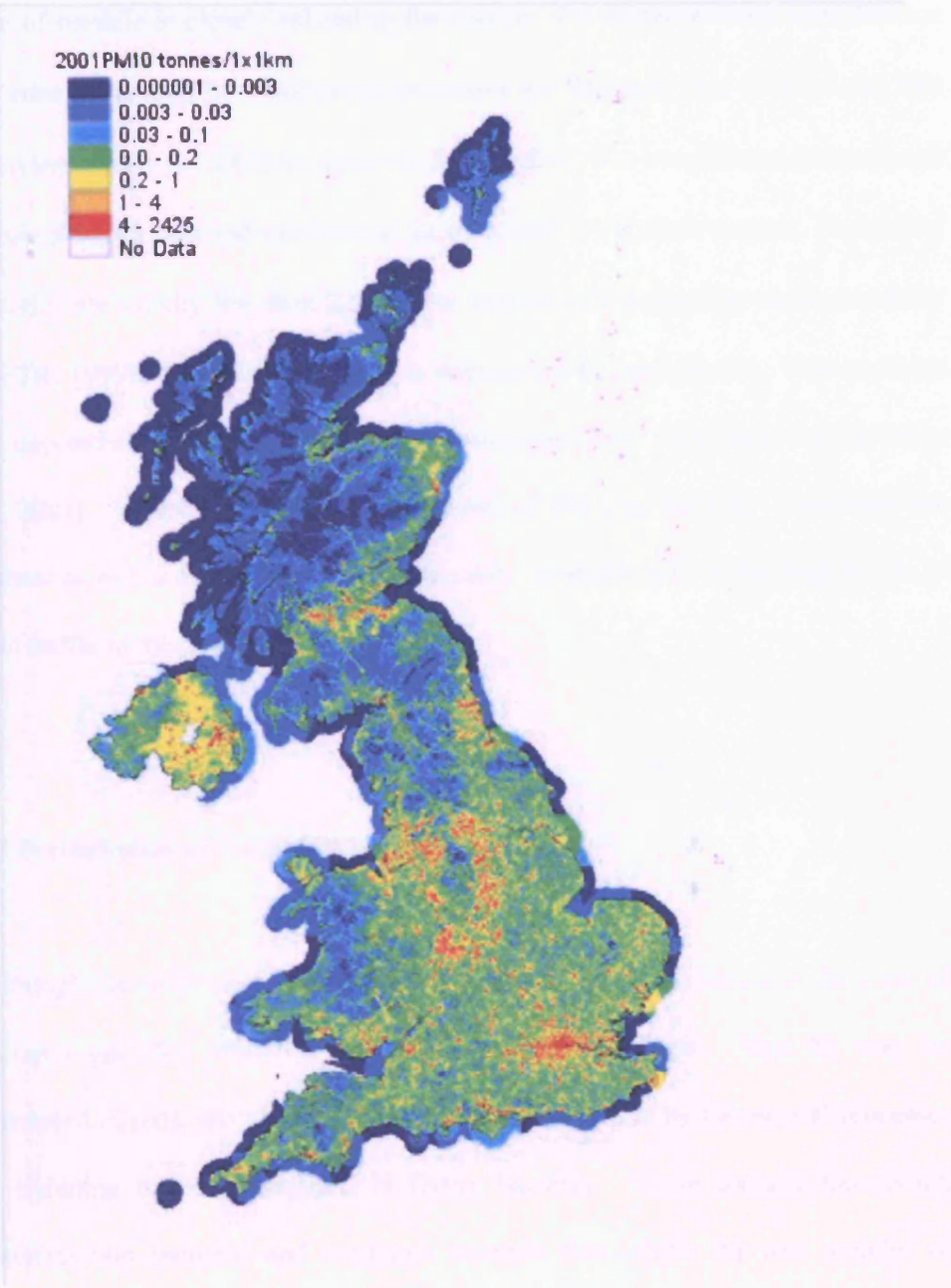


Figure 1.3. Emission map for PM_{10} particles during 2001. From National Atmospheric Emissions Inventory (www.naei.org.uk)

Size of particle is closely related to the source. For instance those particles that are emitted directly by combustion processes are generally less than 2.5 μm and often less than 1 μm whereas those originating from non-combustion sources such as, wind blown dust and mechanical attrition tend to be much coarser. Secondary particles are usually less than 2.5 μm but size do vary depending on the humidity (DETR, 1999). Deposition velocity is determined by particle size. Fine particles are deposited slowly and hence can be transported over long distances (Allen *et al.*, 2001). Figure 1.3 shows the emissions of PM_{10} in the UK. Emissions are highest in and around major towns and cities, probably due to the high levels of road traffic in these areas.

1.3 Perturbation of the global N cycle

Although the earth's atmosphere consists of nearly 80% N, this is in the form of nitrogen gas (N_2) which is largely unavailable to plants. This N_2 may be converted (fixed) into plant available (reactive) N forms by the natural processes of lightning or by specialised N fixing bacteria. These include free-living, nonsymbiotic bacterial and symbiotic bacteria that inhabit the root nodules of legumes and certain other plants.

In the modern world N_2 may also be converted into reactive N forms such as NO_2 and NO by combustion processes (section 1.2.1). Consequently, whereas in the absence of pollution, the N cycle is a highly regulated system with N losses

balancing N inputs, in the polluted world, additional N fixation is causing severe disruption of the cycle (Vitousek, 1994). It is estimated that human activities contribute more 'fixed' N forms than natural processes, with anthropogenic sources contributing 140 Tg N yr^{-1} compared to the 130 Tg N yr^{-1} that are fixed through natural processes (Galloway *et al.*, 1998).

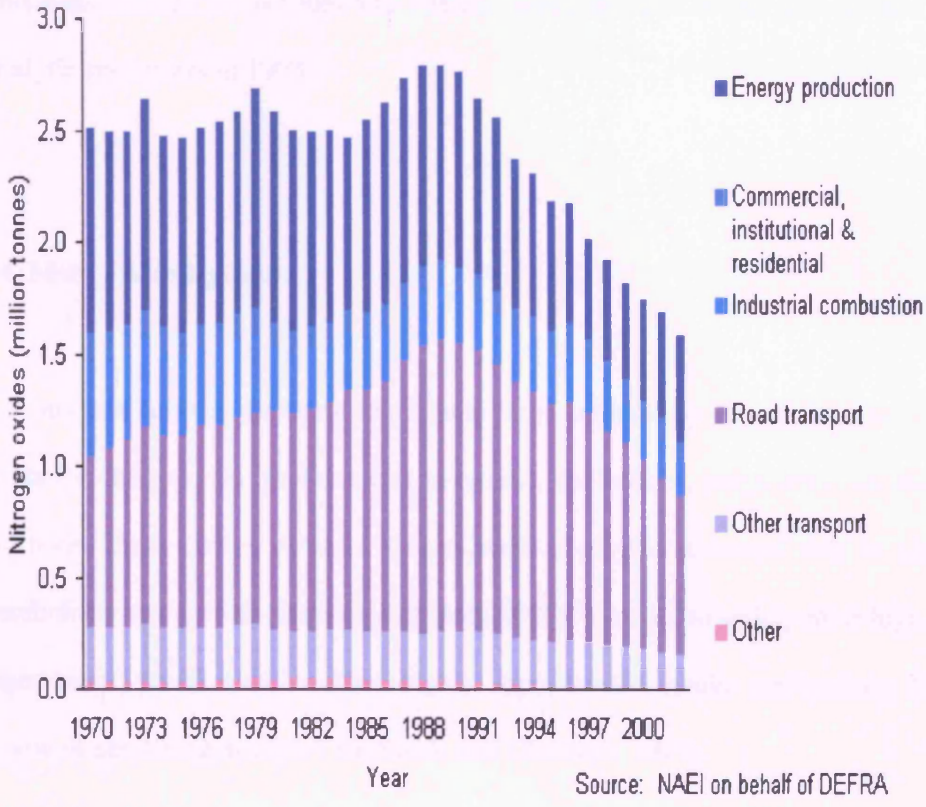


Figure 1.4. Nitrogen oxide emissions and sources in the UK since 1970. Graph obtained from National Atmospheric Emissions Inventory (NAEI) at www.aeat.com

Reactive atmospheric nitrogen forms are now recognized as one of the most ecologically important pollutants. These include reduced N in the form of ammonia and oxidized N (NO_x). Figure 1.4 shows the annual emissions of NO_x from various sources in the UK between 1970 and 2004. Emissions reached their peak in 1989 following a steady increase in the use of road transport in the UK. From 1990 onwards however emissions have declined due partly to changes in energy production and as well as European initiatives to reduce transport emissions through tighter fuel and vehicle standards such as the introduction of catalytic converters in 1993.

1.4. Nitrogen and plants

N is an essential macronutrient required in large amounts by plants. It is used by plants for amino acids (proteins and enzymes), nucleotides, porphyrins, alkaloids and some lipids (Allen *et al.*, 1974) as well as playing a central role in plant metabolism and growth (Haynes and Goh, 1978). In order to satisfy their high N requirement, plants have evolved highly sophisticated uptake mechanisms (for review of uptake mechanisms see Forde and Clarkson, 1999).

1.4.1. Nitrogen availability and preference

The N form and availability are two of the most important factors influencing the species composition of an ecosystem. Habitats can differ markedly with respect to the dominant form of available N. In ecosystems such as pine forests and

wetlands, NH_4^+ is the main N form (Drake *et al.*, 1996; Finzi *et al.*, 2002) while in grasslands, temperate agricultural regions (Shaw *et al.*, 2002) and areas of disturbance NO_3^- dominates.

Consequently, species commonly found in such habitats often show a preference for either of these two inorganic N forms. The preference of tree species for either N form may be determined by measuring NO_3^- reduction within the plant or by examining uptake and assimilation of ^{15}N-labelled NO_3^- and NH_4^+ (Smirnoff *et al.*, 1984; Clough *et al.*, 1989; Stewart *et al.*, 1992; Clough, 1993; Pearson and Soares, 1995).

It was discovered that pioneer species usually have a preference for NO_3^- while climax species have a preference for NH_4^+ uptake (Clough, 1993). For the context of this thesis pioneer species refer to secondary colonisers in a succession, where disturbance or the move to a natural climax community has reverted. Another characteristic of pioneer species, aside from a preference for NO_3^- as an N source, is the tendency for N assimilation to take place in the shoots. Assimilation of NH_4^+ by the roots of climax species is necessary since NH_4^+ is toxic (Marschner, 1995). Pioneers and climax species also differ with respect to their ability to form mycorrhizal associations as climax species will form these associations far more readily than pioneers (Grime *et al.* 1988).

The differing characteristics of pioneers and climax species mean that they are adapted to different habitats. Whereas pioneers grow best in unshaded locations with a high NO_3^- availability such as forest gaps or margins, climax species grow

well in established woodlands where NH_4^+ is the dominant N source and there is greater shading from other trees. High light availability affords pioneer species with the opportunity for the high photosynthetic activity necessary to support shoot N assimilation with regards to C skeletons. Conversely, the restriction of N assimilation to the roots in climax species may allow greater control over use of limited light between N and C assimilation (Smirnoff and Stewart, 1985).

1.4.2. Nitrogen assimilation

Although plants may differ with respect to their preference for N form and location of assimilation, be it in the roots or the shoots, all plants possess the same apparatus for N assimilation. Inside the plant cell the stages of N assimilation are as follows: where NO_3^- is the N source the enzyme nitrate reductase (NR) converts this into nitrite (NO_2^-) (reaction 1.5), which is quickly converted to NH_4^+ by the enzyme nitrite reductase (NiR) (reaction 1.6).

$$NO_3^- + NAD(P)H + H^+ \rightarrow NO_2^- + NAD^+ + H_2O \text{ (NR)} \quad (1.5)$$

$$NO_2^- + 6\,e^- + 8H^+ \rightarrow NH_4^+ + 2H_2O \text{ (NiR)} \quad (1.6)$$

NH_4^+ formed from this reaction or taken up directly from the soil is then converted to amino acids (AA) by the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway (Dennis *et al.*, 1998).

1.5. Nitrogen cycling between plants and soil

Plant's high requirement for N, coupled with the relatively low levels of this element in the soil, means that once taken up, N is highly conserved (Vitousek and Howarth, 1991). Plants usually recover a large proportion of N before leaf fall and therefore the N content of leaf litter is very low. According to Pearson *et al.* (2002) 50-70% of N is recovered at senescence. Killingbeck (1996) estimate the C:N ratio of recent leaf litter to be between 100-200 compared to around 50-100 for live plant material.

In the absence of anthropogenic inputs the terrestrial N cycle is a tightly regulated system with inputs balancing outputs. Soil organic matter (SOM) is the largest N pool, accounting for up to 90% of the total ecosystem N (Temperton *et al.*, 2003). It consists of recalcitrant components of plant detritus and dead microbes. SOM is generally not available to plants since most plants have a preference for inorganic N.

N cycling within the soil is shown in Figure 1.5. Soil microbes convert the N in SOM into inorganic N. This process is known as mineralisation. Ammonifying bacteria produce NH_4^+ which is then available to be converted to NO_3^- by the nitrifying bacteria. Both plants and microorganisms take up NO_3^- and NH_4^+.

N can become immobilised within the soil because the majority of N within the microbial biomass is returned to SOM following microbial death. This is referred to as the microbial N loop. As a result, the decomposition of SOM occurs slowly

and creates a bottleneck in the N cycle. The rate of SOM decomposition is indirectly dependent on plants. Whereas microbes acquire most of their N from SOM, C is obtained from plant exudates and leaf litter. It is the supply of C from plants that determines the net rate of mineralization and immobilization (Knops *et al.*, 2002)

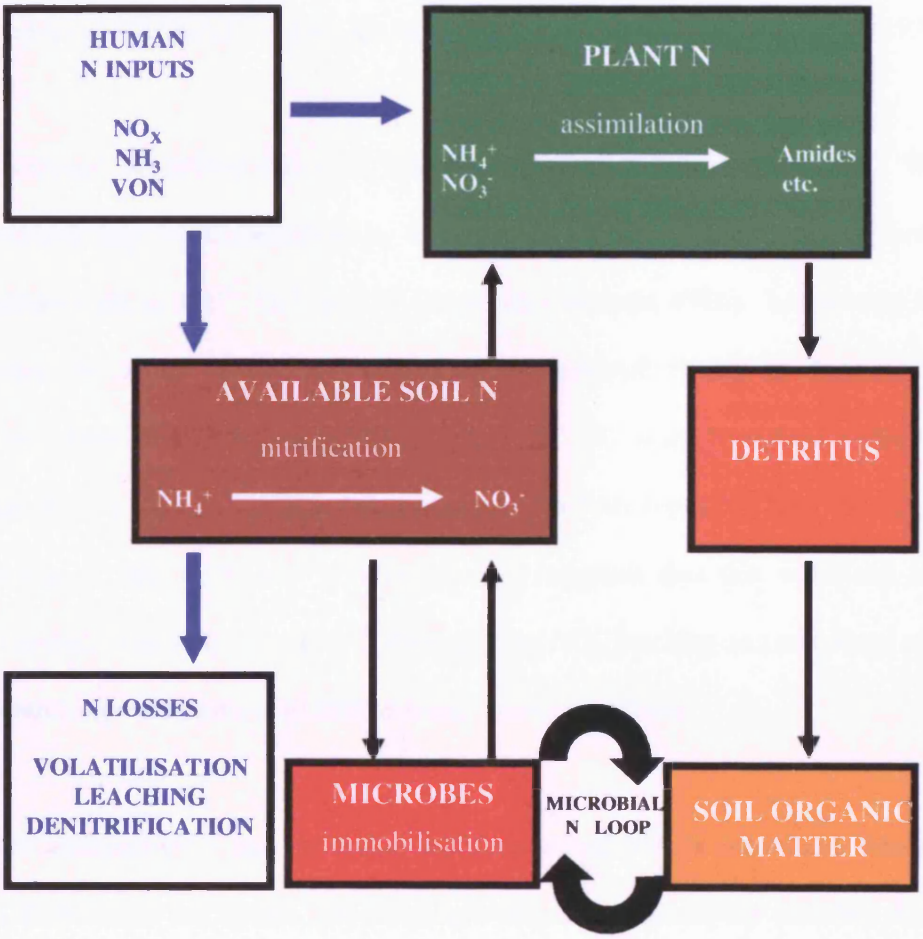


Figure 1.5. Nitrogen flow within ecosystems. Black arrows indicate flow of N between soil and plants. Blue arrows indicate inputs and losses of N from the ecosystem.

1.6. Impact of N deposition on below ground processes

N deposition has a wide range of effects on soil processes. Most obviously it increases the soil N content. N availability is increased further by the stimulation of mineralisation (Fisk and Schmidt, 1996; Wedin and Tilman, 1996; Lee and Caporn, 1998; Falkengren-Grerup *et al.*, 2004) and this effect on mineralisation is long-lived (20 years) and may not be readily reversible (Vinton and Burke, 1995)

Continued deposition can also lead to soil N saturation and leaching. NO_3^- leaching may be accompanied by the removal of equivalent amounts of mobile cations such as Mg^{2+}, Ca^{2+} and K^+ (Reuss and Johnson, 1986). Losses may also occur by increased denitrification. At Rothamsted fluxes of N_2O to the atmosphere have been measured at 1.5 kg N_2 ha^{-1} yr^{-1} in the Broadbalk wilderness (Goulding *et al.*, 1998). This rate compares with those for arable land receiving in excess of 200 kg N ha^{-1} yr^{-1} and strongly suggests that this woodland is N saturated. Similarly, Vitousek (1994) reported NO_3^- leaching and emissions of N-containing trace gases (e.g. NO) in many forest ecosystems.

Soil acidification is another major factor associated with N deposition. Research at Rothamsted has shown that soils have become progressively acidified during the last 110 years (Goulding *et al.*, 1998). In the long-term Park Grass experiment soil pH has decreased from 7 to 4 in the absence of experimental N additions. This is thought to be related to the increase in N deposition from anthropological sources since the start of the experiment. Within soils specific buffering mechanisms operate for different pH ranges (Schulze, 1989). Between pH 8.6

and 6.2 calcium carbonate is the principal buffer. If the pH is between 6.2 and 4.2 cations on clay minerals are exchanged for H^+. Long term acidification leads to the leaching of base cations from the soil as well as a loss of buffering capacity. At low pH, Al and other toxic metals become mobilized (Sverdrup *et al.*, 1995). Aluminium is present in large quantities in most soils however in non-acid soils it is not very soluble. As acidity increases so does Al solubility. Not only is Al toxic to plants but it also suppresses the uptake of Mg^{2+} ions (Schulze, 1989; Sverdrup *et al.*, 1995).

Soil pH is also a controlling factor in the amount of nitrification / denitrification carried out by soil microbes. Nitrification is stimulated by high pH with the result that NH_4^+ predominates in acid soils and NO_3^- is more common in more alkaline soils (Falkengren-Grerup *et al.*, 1998). The combined effects of N deposition and acidification of soils as well as numerous secondary effects such as the leaching of nutrients, changes in soil microbial activity and increases in the availability of toxic metals can have serious impacts on plant productivity and health (Bobbink and Lamers, 2002)

1.7. Impact of nitrogen deposition on vegetation

Whilst extra N may benefit low input farming systems and modern crop varieties with a high N requirement, there may be serious impacts on the species diversity in natural and semi-natural ecosystems. The combination of eutrophication and acidification that accompany N deposition can have serious impacts on plant

health. These two phenomenon are usually positively correlated, making it difficult to separate their effects on plant growth. (Diekmann *et al.*, 2002).

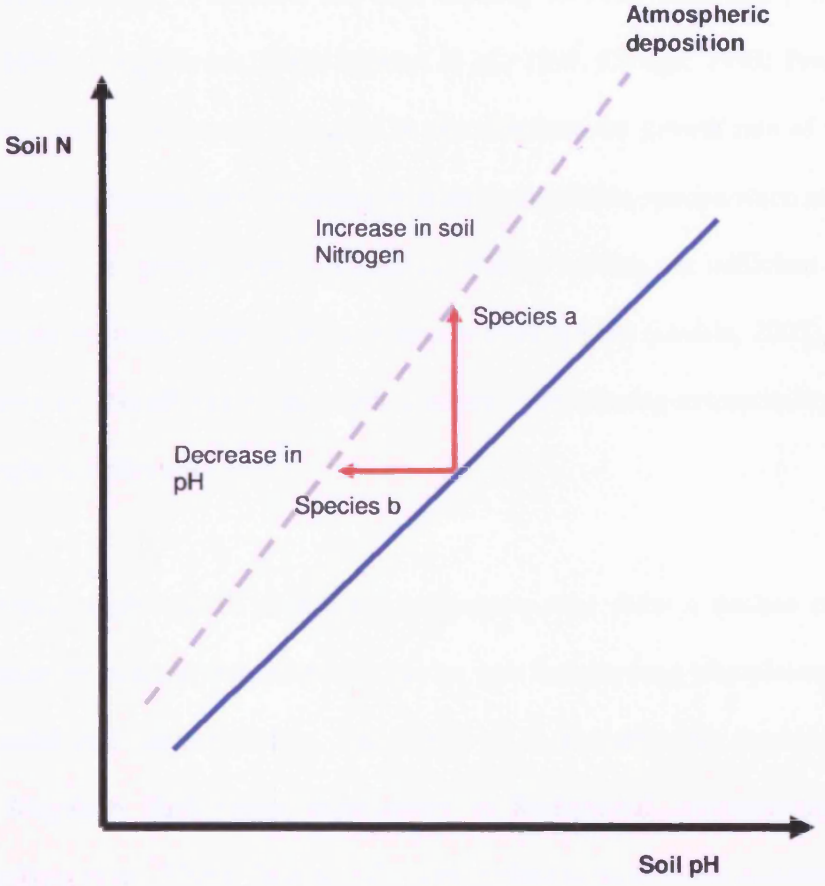


Figure 1.6. Theoretical relationship between soil N availability and pH and its response to eutrophication and acidification (grey line). Species a and b have ecological optima to the left of or above the line and are therefore favoured (from Diekmann *et al.*, 2002).

Plant species A and B in Figure 1.6 are favoured by N deposition because they are naturally adapted to the conditions arising as a result of N deposition. Species A and B can be termed nitrophilous species as they have a high capacity for N uptake and assimilation. Many pioneer species are considered to be nitrophilous due to high foliar N contents and high capacity for NO_3^- reduction (Smirnoff *et al.*, 1984; Clough *et al.*, 1989; Stewart *et al.*, 1992; Clough, 1993; Pearson and Soares, 1995). Although increased N can stimulate the growth rate of the more nitrophilous species, this frequently is at the cost of other species since even small differences in growth rates between competing species are sufficient to bring about the eventual competitive exclusion of most species (Loehle, 2003). High N inputs may also affect the competition indirectly by altering susceptibility to frost, drought or herbivory.

Following prolonged N deposition ecosystems may show a decline in species diversity or become dominated by one or two fast-growing nitrophilous species (Bobbink and Lamers, 2002). The effects of N deposition are demonstrated by the long-term Park Grass experiments at Rothamsted Experimental Station (Goulding *et al.*, 1998). Where NO_3^- was applied to plots in the concentration of 48 Kg N ha^{-1} yr^{-1} species number dropped from 50 to 35 within a few years. The effects were even more extreme when N was applied in the form of NH_4^+ due to the greater shift in soil pH. Changes in species composition have also been observed in a range of semi-natural environments. For example, N deposition has been implicated with (i) the replacement of *Erica* by *Molinia* in wet heaths in the Netherlands (Aerts and Bobbink, 1999) (ii) a decline in original *Sphagnum* moss species and an invasion by *Betula pubescens* and *Molinia caerulea* in a

raised bog in Denmark (Bobbink and Lamers, 2002) (iii) and the increase in a range of nitrophilous understory species in a forest ecosystem in north-east France (Thimonier *et al.*, 1994).

1.8. Air Quality Standards

In order to measure and quantify the potential ecological damage that pollution may have on semi-natural habitats, the United Nations Economic Commission for Europe (UNECE) developed the concept of 'critical loads'. Critical loads are defined as, 'a quantitative estimate of the exposure to one or more pollutants below which significant harmful effects on specified sensitive elements of the environment do not occur according to present knowledge' (Nilsson and Grennfelt, 1988). In other words they are the level of pollutant below which there is no effect on plant's vigour health or productivity. So far, critical loads have been established for the pollutants, SO_2, NO_x, ammonia (NH_3) and ozone (O_3) (Sanders *et al.*, 1995). For N, critical loads have been set for a number of grassland, heathland and forest ecosystems based on the results of experimental studies, field observations or according to 'expert judgement'.

Until recently critical loads for N were set at 7-20 and 10-20 Kg ha^{-1} yr^{-1} for coniferous and deciduous forests respectively (Bobbink *et al.*, 1996). However at the recent Berne critical load workshop (Acherman and Bobbink, 2003) it was decided that the critical load would be set for all forest types at 10-20 kg N ha^{-1} yr^{-1} with the recommendation that national experts use broad guidance to modify

these values for national mapping purposes. Three techniques of assessing UK forest critical loads were recommended depending on the forest type. These are (i) measurements of leached NO_3^-, (ii) changes in ground flora, lichens and algae and (iii) the mass balance approach, involving the calculation of long-term inputs and outputs. Falkengren-Grerup and Diekmann (2003) previously called for the subdivision of critical loads in order to take into account subtypes of deciduous forests, dominating tree species and soil type. These authors found the effect-related critical load to be around 7-10 Kg N ha^{-1} yr^{-1} for deciduous forests in Southern Sweden with the most pronounced effects occurring in forests with low soil pH. However, at the Berne critical loads workshop , critical loads for forests were neither lowered nor subdivided on the basis that N deposition was underestimated or that climate and other pollutants may have been important factors in these observational studies.

1.9. Aims and objectives

Port and Thompson (1980) observed elevated N content and frequency of insect attacks in trees growing alongside a major UK motorway. Aside from this and a few other studies that have concentrated primarily on the effect of Pb (Pearson *et al.* 2000), very little work has specifically examined the effects of traffic pollution and NO_x on vegetation.

This project is based mainly on fieldwork conducted at a small woodland downwind of a major road. This site was chosen to allow us to investigate the

effects of atmospheric N on a range of tree species along a transect running downwind from the motorway. The first objective was to asses the contribution of traffic-derived NO_x to N deposition at a deciduous woodland adjacent to a motorway and to establish whether deposition is having a fertilising effect on plant growth. The second objective was to investigate the effects of increased N deposition on foliar uptake and tree physiology. The last aim was to investigate the deposition of Zn particles and NO_3^- to leaf surfaces as a technique for monitoring traffic exposure.

This work is important because it may help predict future effects of NO_x on roadside trees. In addition findings may also prove useful to those responsible for planting along motorway verges and around other point sources. For example, in the future roadside plants may be selected based not only on their tolerance to pollution but on their ability to cleanse the atmosphere. A further reason to look at this topic is to identify easily measurable changes in plants that may be used as direct biomarkers for traffic pollution.

1.10. Thesis overview

This thesis comprises seven chapters. The first introduces the background to the work while the second details the materials and methods used in conducting fieldwork. The third chapter reports on the deposition of motorway derived NO_x to roadside trees at our field site as well as using total foliar N, stable isotope of nitrogen (^{15}N), and growth measurements to estimate the contribution of

atmospheric reactive N to plant N status. The fourth chapter describes the effects of N deposition on tree physiology and seasonality. The fifth chapter focuses on NR enzyme and how this may be used as a biomonitor for N deposition. The sixth chapter details the deposition of pollutants to roadside leaf surfaces. The final chapter reviews the experimental data and relates the results to the current understanding of the impacts of atmospheric N deposition on woodlands.

CHAPTER 2

MATERIALS AND METHODS

2.1. Chemicals

All chemicals were obtained from BDH Chemicals Ltd. (Poole, England) and Sigma Chemical Company (London, England) unless stated otherwise.

2.2 Experimental sites

2.2.1. Mardley Heath

Plant material was collected from Mardley Heath in Hertfordshire (grid reference TL245185). This small woodland is adjacent and downwind to the AI Motorway. The high volume of traffic on the busy road means that it is a potential point source of atmospheric nitrogen oxides (NO_x). Sampling was conducted at 2 sites at either end of a transect running in an easterly direction from the motorway to the control site 600 m downwind (Figure 2.1). Transects from point sources allow workers to demonstrate spatial associations between pollutant exposure and response. Air at the control site is unlikely to be completely pollutant free but is likely to contain significantly less NO_x than at the motorway end of the transect. It has been established that NO_x concentrations decrease significantly away from the immediate vicinity of the road (The Air Quality Strategy, 1999)

Figure 2.1. Ordnance survey map showing Mardley Heath sampling site and surrounding area. The transect is marked on the map in black. The letters a and b mark the locations where 'motorway' and 'control' material were collected respectively. *Image produced from the Ordnance Survey get-a-map service. Image reproduced with kind permission of Ordnance Survey and Ordnance Survey of Northern Ireland.*

Sampling at Mardley Heath took place approximately once a month during the growing season of both 2001 and 2002. Three individuals of each species investigated were sampled at both sites. Sampling was always performed at a

similar time of day, approximately 11 am. Leaf material was collected from branches approximately 1.5 m in height and was packaged in a cool bag for transfer to the laboratory for analysis. Xylem sap was extracted from cut branches using a Millipore pump at 25-30 KPa vacuum pressure. Sap was collected into 1 ml Eppendorfs and frozen for later analysis.

2.2.2. Marylebone Road

For the investigations into surface contaminants, leaf material was occasionally collected from roadside trees along Marylebone road (grid reference TQ286821). This is one of the busiest roads in central London. A pollution-monitoring site is located along Marylebone road and this station measures a range of pollutants including PM_{10} and NO_x concentrations.

2.3. Species

Several tree species were initially investigated including *Betula pubescens* Ehrh., *Quercus spp*, *Sambucus nigra* L. and *Crataegus monogyna* Jacq.. From 2002 onwards research was concentrated on two species, *S. nigra* and *C. monogyna*, to enable more detailed biochemical analysis to be performed. These two species were selected because of their abundance at both sampling sites and to allow comparison between species that have different N metabolism strategies (Soares *et al.*, 1995). *S. nigra* utilises NO_3^- as its N source and performs most of the assimilation in the shoots while in *C. monogyna* there is less emphasis on foliar

assimilation of NO_3^-. *C. monogyna* can utilise both NO_3^- and NH_4^+ and carries out a large proportion of assimilation in the roots (Clough, 1993).

2.4. Weather information

Information regarding prevailing weather conditions at the Mardley Heath field site was obtained from the Iceni weather station (www.iceni.org.uk). This weather station is located in Royston, Hertfordshire and is approximately 16 miles from the field site.

2.5. Assessment of tree growth

Tree growth was measured towards the end of the growing season. Growth was assessed by measuring the length of branch extension and number of new leaf nodes for the current year. Measurements were taken on 15 branches of 5 trees at each field site.

2.6. Determination of nitrogen dioxide deposition

Nitrogen dioxide deposition at Mardley Heath was investigated using NO_2 diffusion tubes (Gradko, Winchester, UK). These tubes were attached to either fence posts or tree stems along a transect at Mardley heath that ran from 10 m

upwind of the motorway to 600 m downwind. Two tubes were used as blanks and these remained sealed in a plastic container during the exposure period. After 14 days all the tubes were collected and analysed in the laboratory. The absorbent discs were carefully removed and eluted in 2 x 0.5 ml dd H_2O for 2 minutes. The combined eluted solution (1 ml) was assayed for NO_2^- concentration by adding 1 ml 1% w/v sulphanilamide in 1 M HCl and 1 ml 0.02% N-napthyl ethylene diamine dihydrochloride (NEDD) in dd H_2O. The mixture was shaken and the colour was allowed to develop for 20 minutes. The absorbance of each sample was measured against the blank (reagents with no nitrate) using a Beckman DU-7 Spectrophotometer set at a wavelength of 540 nm. The NO_2 deposition was estimated by using a calculation based on deposition velocity to woodlands of 43.5 mm s^{-1} (Fowler, 2002).

2.7. Enzyme assays

Enzyme assays were carried out on fresh leaf material. Activities were determined within 1 hour of collection from field sites. Enzyme extraction from plant material was carried out at 4°C. Incubation conditions for the enzyme assays consisted of a water bath maintained at 25°C. In establishing enzyme activities for new species, initial tests were carried out with different volumes of extract and for different lengths of time to check for linearity and proportionality of enzyme activity.

2.7.1. Nitrate reductase (E.C.1.6.6.1) activity in vivo

Nitrate reductase activity (NRA) was measured using the *in vivo* method of Stewart and Orebamjo (1979). Approximately 0.1g of finely divided leaf tissue was weighed out and added to a small vial of 5 ml assay medium. The assay medium consisted of 0.1 M phosphate buffer at pH 7.5, 150 mM (1.5%) KNO_3 and 1.5% v/v propan-1-ol. The vials were then placed into a desiccator and vacuum infiltrated for 2-3 minutes using a Millipore pump at 25-30 KPa during which time the vacuum was released several times. Vials were then incubated at 25°C in the dark for a 1 hour period. Following incubation the vials were shaken slightly before 1 ml assay medium was removed and placed into a test-tube. To this, 1 ml 1% w/v sulphanilamide in 1 M HCl was added followed by 1 ml 0.02% N-napthyl ethylene diamine dihydrochloride (NEDD) in dd H_2O. A blank was made by substituting 1 ml fresh assay medium for 1 ml incubated assay medium. The mixture was shaken and the colour was allowed to develop for 20 minutes. The absorbance of each sample was measured against the blank using a Beckman DU-7 Spectrophotometer set at a wavelength of 540 nm. NR activity was calculated using the slope for the standard curve.

2.7.2. Nitrate reductase (E.C.1.6.6.1) activity in vivo – plus and minus KNO_3

The above assay method allowed the estimation of NRA_{KNO3}, a measure of NO_3^- reduction capacity at non-limiting NO_3^-. NRA_{H2O} as a measure of the actual NR activity (Andrews, 1986; Keltjens and van Loenen, 1989; Thomas and Hilker,

2000) was determined by the above method but without the addition of 1.5% KNO_3 to the assay medium.

2.7.3. Nitrate reductase (E.C.1.6.6.1) activity in vitro with Zn additions

Determination of *in vitro* NR activity depends on the careful extraction of intact enzyme. Approximately 0.2 g fresh leaf tissue was weighed out and homogenised in a pestle and mortar with liquid N, sand and Polyvinyl polypyrolidone (PVPP). Extraction buffer at pH 8.5 was added to the leaf powder and spun at 4°C for 20 minutes. The extraction buffer contained 0.1 M Tris, 1 mM Na_2EDTA, 20 µM Leupeptin, 10 mM DTT, 1 mM $NaMoO_4$, 10 µM FAD and 0.1% w/v nonidet detergent and this could be stored at 5°C for 1 week. 0.1% v/v mecaptoethanol was added on the day of use. The supernatant was decanted off and 100 µl was added to an 1 ml Eppendorf tube along with 300 µl phosphate buffer at pH 7.5, 50 µl 0.1 M KNO_3 and 50 µl 10 mM NADH. To test the effect of Zn on the enzyme, $ZnCl_2$ was also added to the buffer. A blank was made by substituting the enzyme extract with 100 µl extraction buffer. Eppendorfs were incubated in a water bath at 25°C for 20 minutes and the reaction was terminated by the addition of 0.5 ml 1% w/v sulphanilamide in 1 M HCl. Then 0.5 ml 0.02% w/v N-napthyl ethylene diamine dihydrochloride (NEDD) in dd H_2O was added and a pink colour was allowed to develop for 20 minutes. The absorbance of each sample was measured against the blank using a Beckman DU-7 Spectrophotometer set at a wavelength of 540 nm. NR activity was calculated using the slope for the standard curve.

2.8. Acid digestion and element determination

In order to determine total leaf concentrations of N, P, and Zn leaf material was first digested in a sulphuric acid – hydrogen peroxide mixture. This reagent was prepared using a method adapted from Allen *et al.* (1974). Selenium (0.42 g) and lithium sulphate (14.0 g) were dissolved in 420 ml concentrated sulphuric acid and 350 ml hydrogen peroxide on ice. 0.05-0.2 g of oven-dried sample was accurately weighed and placed in thick-bottomed boiling tubes with 4.4 ml of digestion reagent. The mixture was heated gradually to a temperature of 360°C over a 2-3 h period in a heating block (Grant Instruments, Cambridge Ltd.), until the digest had cleared. The digest was allowed to cool before being made up to 50 ml with dd H_2O. This diluted digest was used in the determination of total N, total Zn and total PO4.

2.8.1. Total nitrogen determination

Before total N analysis could be carried out, a 0.5 ml aliquot of the digest solution was neutralised by adding 0.5 ml 1.5 M NaOH and 4.0 ml 0.2 M phosphate buffer at pH 7. This solution could then be analysed using the method of McCullough (1967). Solutions A and B were prepared as follows:

Solution A: 10 g phenol
50 mg sodium nitroprusside
made up in 1 l of dd H_2O

Solution B: 5 g NaOH

53. 7 g $Na_2HPO_4.12H_2O$

10 ml sodium hypochlorite solution (10-14% v/v)

made up in 1 l of dd H_2O

2.5 ml of solution A was added to 0.5 ml of neutralised digested sample and mixed well. To this mixture, 2.5 ml of solution B was added before incubation at 37°C for 40 minutes. The optical density was read at 625 nm using an Amersham Biosciences Ultrospec 3100 pro spectrophotometer. For blanks, assays were performed in an identical way but without the addition of plant material to the digest solution. $(NH_4)_2SO_4$ was used to create a standard curve.

2.8.2. Total phosphorus determination

Molybdate-antimony solution was prepared as described by Golterman (1978). 0.02 g of sodium antimony tartrate ($NaSbOC_4H_4O_6$) and 0.96 g of ammonium molybdate ($(NH_4)_6Mo_7O_{24}.4H_2O$) were dissolved in 100 ml 2M H_2SO_4 and made up to 500 ml with the same acid. Ascorbic acid solution was prepared by dissolving 2.0 g ascorbic acid in 100 ml dd H_2O. The molybdate-antimony solution (1 ml) and the ascorbic acid solution (0.5 ml) were added to 10 ml digested sample and mixed well. The mixture was incubated at 37°C for 30 min. The optical density was read at 882 nm using an Amersham Biosciences spectrophotometer, model Ultrospec 3100 pro. A standard of KH_2PO_4 was used to calibrate this method.

2.8.3. Total zinc determination

Total Zinc was measured using a Pye Unicam SP9 atomic absorption spectrophotomer. Calibration standards containing $ZnCl_2$ were prepared in a solution of the same composition as that of the digest mix.

2.9. Tissue extraction

Tissue was extracted by placing 0.2 - 0.5 g finely chopped tissue into vials containing 5 ml methanol. Vials were stored in sealed tubes for 1 - 3 days at 4°C.

2.10. Determination of tissue and xylem nitrate

Nitrate content was determined enzymatically using nitrate reductase (NR) from the fungus, *Aspergillus nidulans*. Where tissue nitrate was being assayed, the extraction solution (section 2.11) protein was first removed. Protein was removed by adding 0.2 ml $K_4Fe(CN)_6$ (3.6 g in 100ml ddH_2O) to 0.8 ml of extraction solution before mixing, then adding 0.2 ml $ZnSO_4$ (7.2 g in 100 ml ddH_2O) and spinning for 10 minutes at 13,000 rpm.

5 -100 µl of sample (either xylem sap or tissue extraction solution with protein removed) was added to 370 µl 0.1 M phosphate buffer at pH 8. To this,10 µl each

of NADPH (13 mmol/l), FAD.Na_2 (0.23 mmol/l) and NR (4.5 U/ml) were added. dd H_2O was added to make the volume up to a final volume of 500 µl. A blank was made by using either 100 µl methanol or dd H_2O depending on whether the assay was being carried out on tissue extraction solution or xylem sap. The samples were incubated for 1.5 hours in a water bath at 25°C and to the same Eppendorf tube, 0.5 ml sulphanilic acid and 0.5 ml NEDD were then added to stop the enzyme reaction. The mixture was shaken and the colour was allowed to develop for 20 minutes. The optical density of the samples was measured in a Beckman DU-7 Spectrophotometer at a wavelength of 540 nm. A series of standard of KNO_3 solutions was used to create a calibration curve.

2.11. Determination of tissue and xylem soluble amino acids

A volume of 1.4 ml citrate buffer (0.8 M, pH 4.84) and 1.2 ml ninhydrin (1% w/v in dd H_2O) were added to 0.25 – 0.1 ml of extraction solution or xylem sap. The mixture was boiled for 20 minutes then cooled before the addition of 3 ml 60% v/v of ethanol. The optical density that developed in each sample was read on an Amersham Biosciences spectrophotometer (model Ultrospec 3100 pro). Leucine was used as the standard solution for calibration and blanks were prepared by substituting sample with methanol or dd H_2O depending on whether the assay was performed on tissue extraction solution or xylem sap respectively.

2.12. Determination of tissue and xylem sugar content

Sugar content was analysed using a modified method of An and Roel (1993). 25-50 µl of sample (either extraction solution or xylem sap) was first made up to 400 µl in a test-tube with dd H_2O. To this, 2 ml of concentrated H_2SO_4 was added followed by 400 µl of 18% (w/v) phenol (made up in a 30% ethanol solution). This mixture was vortexed vigorously and allowed to stand for 15 minutes. The optical density was read at 490 nm using an Amersham Biosciences spectrophometer (Ultrospec 3100 pro model).

2.13. Determination of ammonia in xylem sap

Ammonia concentrations in xylem sap were assayed using the method of McCullough (1967). A 100 µl sample was added to an Eppendorf tube and to this 600 µl of both reagents 1 and 2 were added. Reagent 1 was prepared by adding 5 g phenol, 25 mg sodium nitroprusside in 500 ml dd H_2O. Reagent 2 was a buffer containing 2.5 g NaOH, 5 ml sodium hypochlorite solution (10-14% v/v) and 21.3 g Na_2HPO_4 made up in 500 ml dd H_2O. Samples were then mixed and incubated in a water bath at 37°C for 40 minutes until a blue colour was observed. The optical density of the samples was measured using a spectrophotometer set to read at a wavelength of 625 nm. A blank for the spectrophotometer readings was made by using 100 µl dd H_2O instead of the sample. Standard solutions containing $(NH_4)_2SO_4$ were used to make a calibration curve.

2.14. Determination of phosphate concentration in xylem sap

Phosphate content was assayed using the method described by Golterman (1978). A xylem sap sample of 100 µl was added to a 1 ml Eppendorf tube. To this, 900 µl dd H_2O was added plus 50 µl molybdate antimony and 25 µl 0.1 M ascorbic acid solution. Molybdate antimony was prepared by dissolving 0.02 g sodium antimony tartrate ($NaSbOC_4H_4O_6$) and 0.96 g ammonium molybdate $(NH_4)_6Mo_7O_{24}.4H_2O$ in 100 ml 2M H_2SO_4. For measuring the samples on a spectrophotometer a blank was made by substituting 100 µl sample with 100 µl H_2O. The Eppendorf tubes were heated at 40°C for 30 minutes. The optical density of the samples was then measured relative to the blank using a Beckman DU-7 Spectrophotometer set at a wavelength of 882 nm. A standard solution KH_2PO_4 was used to create a calibration curve.

2.15. ^{15}N stable isotope analysis using ANCA-MS

Automated 15N/13C analysis – mass spectrometry (ANCA-MS) was used for stable isotope ^{15}N and total N analysis as described by Barrie and Lemley (1989). Samples of plant material were oven dried before being ground to a fine powder using a MM2 ball mill (Retsch GmbH and Co., W. Germany) These samples were placed in 6 mm x 4 mm methanol-washed tin capsules (2-10 µg of sample per capsule). Samples were then analysed for ^{15}N content using ANCA-MS, (Europa Scientific Ltd., Crewe, UK).

2.16. Quantification of leaf surface zinc and nitrate

The surface area of fresh leaves collected from the field was first determined using a hand held, GeniScan leaf scanner. Whole leaves were placed in 20 ml vials, to which 5 ml of either dd H_2O, 0.01 M HCl or chloroform was then added. These vials were shaken vigorously for 2 minutes, after which the leaves were removed, oven dried and weighed. The rinsing solutions were stored at 5°C in a refrigerator prior to analysis.

An atomic absorption spectrophotometer (Pye Unicam SP9 model) was used to analyse the Zn content of the 0.01 M HCl and dd H_2O solutions obtained from the washing procedure. A standard calibration curve was created using ZnCl solutions of varying concentrations. The efficiency of this procedure was checked by increasing the washing times with increments of 30 seconds. This analysis showed that washing for 1 minute removed more than 90% of the Zn deposited on the leaf. NO_3^- was assayed using the method detailed in section 2.10.

2.17. Microanalysis of leaf surface physical characteristics

The scanning electron microscope (SEM) (JEOL 6360LV variable pressure SEM) at Rothamsted was used to study the morphology of epicuticular wax layers on leaf surfaces. Fresh leaf material was always used for the analysis. This was first cut into small sections approximately 5 x 5 mm before being stuck to a specimen

stub. In order to preserve the structure in its original form material was frozen and coated in gold using a cryo-preparation and transfer system (GATAN alto 2100).

2.18. Microanalysis of leaf surface chemical characteristics

The chemical composition of surface particulates was analysed using energy dispersive microanalysis (EDS). This system measures X-rays emitted during electron bombardment in the SEM. By determining the energies of the X-rays emitted from the area being excited by the electron beam, the elements in the sample were determined. The rate of detection of these characteristic X-rays is used to determine the amounts of elements present. The model used was an Oxford instruments INCA 200.

The Oxford instruments INCA 200 consists of a number of important components: an energy dispersive X-ray detector to detect the X-rays, a pulse processor to accurately measure the energy of each X-ray detector, and a software interface to control the system and to analyse the results. Figure 2.1 shows the major components of a standard SEM with EDS facilities.

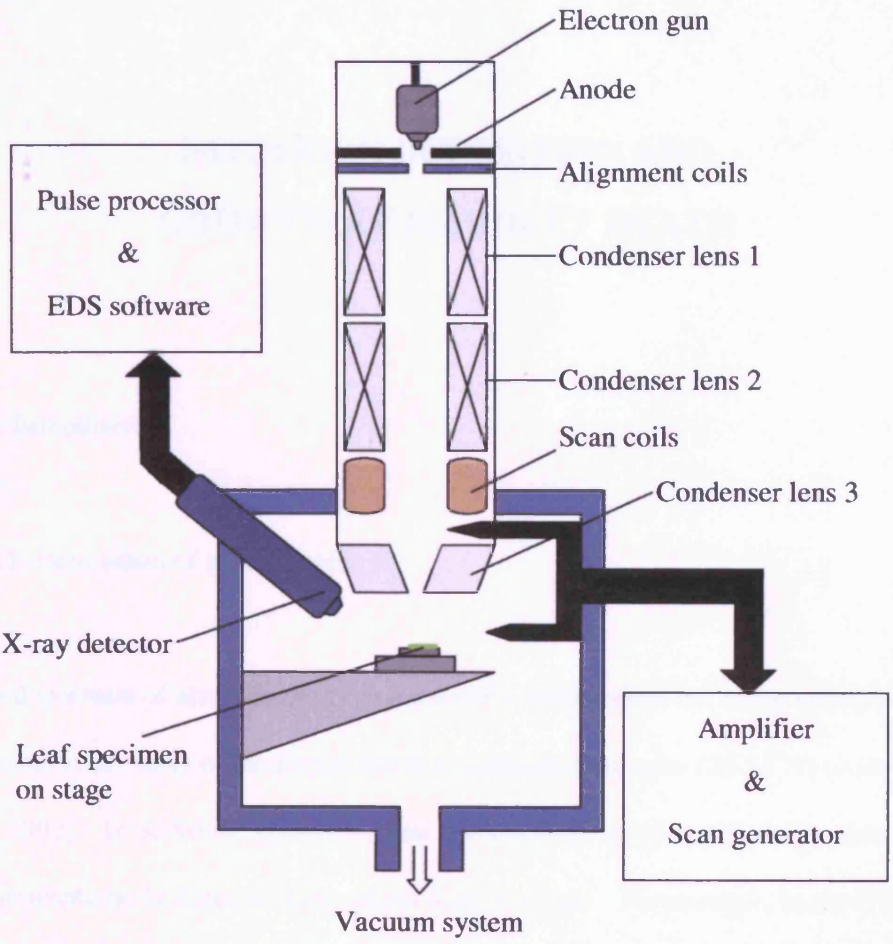


Figure 2.1. Diagram of SEM column showing major components and EDS facilities.

CHAPTER 3

NITROGEN DEPOSITION AND GROWTH AT MARDLEY HEATH

3.1. Introduction

3.1.1. Deposition of atmospheric N

The deposition of atmospheric N to the earth's surface does not occur evenly. On a global scale, most of the deposition is to temperate latitudes (25-55°N) (Asner *et al.*, 1997). In addition, upland regions receive disproportionately large amounts of atmospheric N deposition compared with lowlands. For example, in the UK as a whole, N deposition was estimated at 19 Kg N ha^{-1} yr^{-1} however upland areas are reported to receive 26 kg N ha^{-1} yr^{-1} (Kirkham, 2001). This may be explained by the elevated N concentrations in hill cloud, high levels of precipitation and by the fact that upland areas tend to be very effective sinks for deposition.

Turbulent transfer of atmospheric NO_x and other gases to terrestrial surfaces is dependent on the frictional drag of vegetation. Deposition to woodland is typically higher than to shorter vegetation. Beier and Gundersen (1998) measured N deposition to a forest and found it to be 2.5 times greater than that to

surrounding pasture land. High deposition to forests is thought to be caused by the large frictional drag of tree canopies on airflow (Fowler, 2002). This may explain why at present, temperate forests are receiving nearly all the 5.1 Tg Yr^{-1} of anthropogenic N inputs (Nadelhoffer *et al.*, 1999). Goulding *et al.* (1998) have estimated that deposition to woodland at Rothamsted in Hertfordshire, UK to be in the region of 100 kg ha^{-1} yr^{-1}.

Deposition within forests is also variable. The so-called 'edge effect' describes enhanced N deposition and effects at the forest edge in comparison with the forest interior (Spangenberg and Kölling, 2004). Several other authors have also documented this phenomenon (Draaijers *et al.*, 1988; Cox *et al.*, 1999; Weathers *et al.*, 2000). The most common explanations for this phenomenon are higher wind forces at the forest edge (Veen *et al.*, 1996) as well as increased dry and occult deposition (Beier and Gundersen, 1989).

3.1.2 Effects of N on forest health

Northern Temperate forests have long been considered to be N-limited (Aber *et al.*, 1984; Vitousek and Howarth, 1991). However, where deposition exceeds tree and soil requirements, N saturation can occur. According to Fenn *et al.* (1996) N saturation has been defined as a condition in which:

1. Available N is frequently in excess of total biotic demand
2. Vegetation within the ecosystem no longer exhibits a positive growth response to N addition (Nilsson, 1986)

3. Sustained N losses approximate or exceed N inputs – the N retention capacity of the system has been exceeded.

Recent evidence indicates that forest growth is being enhanced by N deposition. Nasholm (1998) reported that trees experienced few negative effects and were in fact growing vigorously as the result of N deposition. Similarly, Kauppi *et al.* (1992) reported an increase in the growth rate and the amount of growing stock in forests in several European countries.

Stimulated forest productivity may be considered as a positive outcome since it creates a possible sink for excess CO_2 (Neff *et al.*, 2002). However, stimulated growth may be accompanied by nutrient imbalances, increased pathogen attacks and a decline in species diversity (Port and Thompson, 1980; Schulze, 1989; Strengbom *et al.*, 2003). Effects of N deposition on the woodland ground flora have been investigated in Sweden and in N.E. France (Tyler, 1987; Thimonier, 1994; Diekmann *et al.*, 2002). These studies showed strong eutrophication and acidification associated with N deposition as well an increase in nitrophilic species. Such invasions of forest ecosystems by nitrophilous species will occur very slowly since the long life span of trees means they are relatively resistant to displacement (Loehle, 2003). Deposition of N can also disrupt soil mycorrhizae. The effect is dependent on the mycorrhizal type with mychorrhizal infection of roots being inhibited for ectomycorrhizas but variable for vesicular abuscular and ericoid mycorrhizas (Aerts and Bobbink, 1999).

3.1.3. Tree biomarkers for nitrogen deposition

In the establishment of critical loads it is useful to have information concerning the N status and health of the vegetation. Plant biomarkers that allow workers to quickly obtain reliable information regarding effects of prevailing N deposition on vegetation would therefore be very useful to those responsible for setting critical loads (Marsh *et al.*, in press). Total plant N content and δ-15 N signature of plant tissue have both received attention as potential biomarkers.

3.1.3.1. Tree biomarkers for nitrogen deposition: total N

According to Zhen and Leigh (1990) N will accumulate in crop plant tissues beyond the level needed to achieve maximum growth. Many empirical studies have shown a direct relationship between N deposition and foliar N concentrations. This effect has most frequently been demonstrated in ectohydric moss species (Pitcairn *et al.*, 1995). Since mosses depend heavily on the atmosphere to supply nutrients it is thought that these species will be most sensitive to changes in N deposition. Other studies have compared 19th century moss collections with present day moss to show that tissue N has increased over the years in line with increases in anthropogenic N emissions (Baddeley *et al.*, 1994; Pitcairn *et al.*, 1995; Woolgrove and Woodin, 1996).

Correlations between N deposition and foliar N have also been shown in a range of higher plants (Port and Thompson, 1980; Baddeley *et al*, 1994; Pitcairn *et al.*,

1995; Kirkham, 2001). Port and Thompson (1980) found elevated total N concentrations in *Fagus sylvatica* and *Crataegus monogyna* shoots exposed to motorway NO_x pollution. Furthermore, elevated tissue N was implicated with increased invertebrate pest outbreaks in the trees. On a large-scale pollution transect running from N. Sweden to C. Europe foliar N concentration was also shown to rise with increased deposition (Hogberg *et al.*, 1998). Work in the UK has revealed that foliar N concentration was related to N deposition in a range of upland species including *Calluna vulgaris*, *Racomitrium lanuginosum* and *Vaccinium myrtillus* (Baddeley *et al*, 1994; Pitcairn *et al.*, 1995; Kirkham, 2001).

In contrast to the above-mentioned studies, there have been a few investigations where total N content has declined following exposure to NO_2 (Elkiey and Ormond, 1981; Srivastava and Ormond, 1986; Sabaratnam, Gupta and Mulchi, 1988). According to Wellburn (1990) this may be explained by the translocation of additional N from shoots to roots.

3.1.3.2 Tree biomarkers for nitrogen deposition: $\delta^{15}N$ signatures

N is present as two isotopic forms, ^{14}N and ^{15}N of which ^{14}N is by far the most common, accounting for 99.6337% of N_2 in air (Handley *et al.*, 1998). Natural processes tend to have a preference for one of the two forms and hence the ratio of ^{14}N to ^{15}N varies according to where N is sampled within the global cycle. $\delta^{15}N$ is a measure of the difference between the ratio of these isotopes in a sample and in a standard air sample. Plants have a preference for ^{14}N uptake and assimilation

and therefore plant tissues are usually have a negative $\delta^{15}N$. $\delta^{15}N$ values are derived as follows:

$\delta^{15}N$ in $^{o}/_{oo}$ = [(^{15}N / ^{14}N sample - ^{15}N / ^{14}N standard) / ^{15}N / ^{14}N standard] x 10^3

where the internationally accepted standard of N_2 of air is defined at $\delta^{15}N_{air} = 0$ $^{o}/_{oo}$ (Handley *et al.*, 1998).

There is growing evidence that the two main forms of N pollution, as oxidised N and reduced N forms have different stable isotope signatures (Garten, 1992; Heaton *et al.*, 1997). Where anthropogenic N inputs are contributing to plant N, the $\delta^{15}N$ of tissues may be disrupted. It has been proposed that plant tissue $\delta^{15}N$ could be used as a means for monitoring atmospheric pollution in vegetation (Gebauer and Schulze, 1991; Heaton *et al.*, 1997). It is hypothesized that foliar uptake of atmospheric NO_x will be closely related to the source atmospheric signature at the motorway.

3.1.4. Aims and Objectives

This chapter aims to assess of the deposition and contribution of motorway derived NO_x to roadside trees at Mardley Heath. N deposition in the form of NO_2 was estimated along a transect using diffusion tubes. The $\delta^{15}N$ signature was measured in leaves to show the contribution of this oxidised N supply to total plant N. It is generally thought that N does not accumulate in tissues until N

requirements for growth have been met or other essential nutrients become limiting. In order to assess the N status of trees in the woodland we took measurements of foliar N and P concentrations as well as yearly growth. The effectiveness of diffusion tubes as well as plant measurements such as total foliar N, P and annual growth in monitoring N deposition was evaluated.

3.2. Results and Discussion

3.2.1. N deposition within Mardley Heath

NO_x deposition within the woodland was monitored using NO_x diffusion tubes and the results are presented in Figure 3.1. NO_x deposition ranges from 14.8 to 40.7 Kg N ha^{-1} yr^{-1}. These values exceed the current woodland critical loads of 10-20 Kg N ha^{-1} yr^{-1} (Achermann and Bobbink, 2003) and the estimated total annual requirement for trees of 8-12 Kg N ha^{-1} yr^{-1} (Jenkinson, 1971; Pearson and Stewart, 1993).

The maximum NO_x concentrations are found at the woodland edge, just 20 m downwind of the motorway. This is the 'motorway' site where tree material was collected to examine the effects of NO_x on biochemistry and growth. The N elevated deposition was likely to originate from road traffic emissions of NO and NO_2 (sections 1.2.1 and 1.2.2). N deposition concentrations decline as distance from the motorway increases. This decline is likely to be due to the vertical

dispersion of the gas (The Air Quality Strategy, 1999) and deposition to the forest canopy.

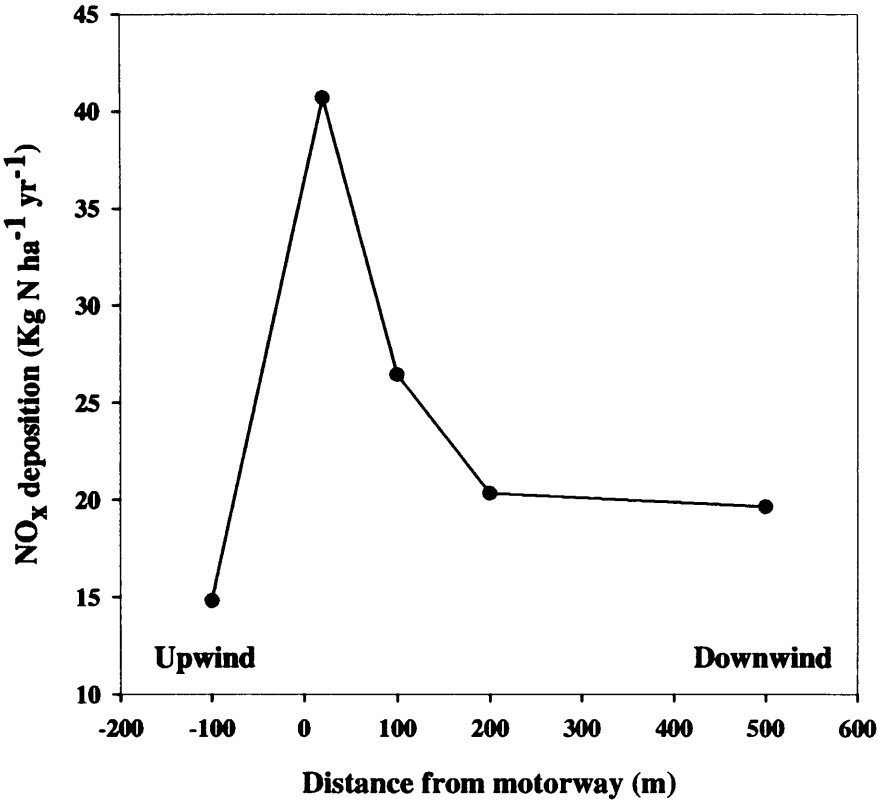


Figure 3.1. NO_2 deposition measured at Mardley Heath. The motorway is located at 0 on the horizontal axis. Diffusion tubes were left at the site for 2 weeks during November 2003.

Other potential contributory sources of NO_x include the Glaxosmithkline Research and Development plant which is located within a 5 km radius of the sampling site and Brazier Methane Conversion plant which is located within 10 km. According to the National Atmospheric Emission Inventory

(www.naei.org.uk), these respectively emit 10 and 273 tonnes of NO_x to the atmosphere each year. Despite these high industrial emissions the majority of NO_x deposition within Mardley Heath can be attributed mainly to road transport which accounts for over 60% of the total emissions from nearby sources (www.naei.org.uk). Industrial emissions, although they may seem large may in fact not be affecting NO_x deposition at the site to a large extent since gases are largely emitted through tall chimney vents.

High concentrations 10 m downwind may also be influenced by the 'edge effect' as discussed in section 3.1.1. (Weathers *et al.*, 2000; Spangenberg and Kolling, 2004). Deposition 500 m from the motorway is in the region of 19.6 kg N ha^{-1} yr^{-1}. This is around the national average of 19 Kg N ha^{-1} yr^{-1} (Kirkham, 2001). At this location, tree material was collected for control measurements. It is clear from these results that the control site is not completely pollutant free. However, the benefit of using this location as a control site rather than a separate clean air site is that other environmental variables such as soil and weather are likely to remain the same while the N deposition is much lower than that at the polluted site.

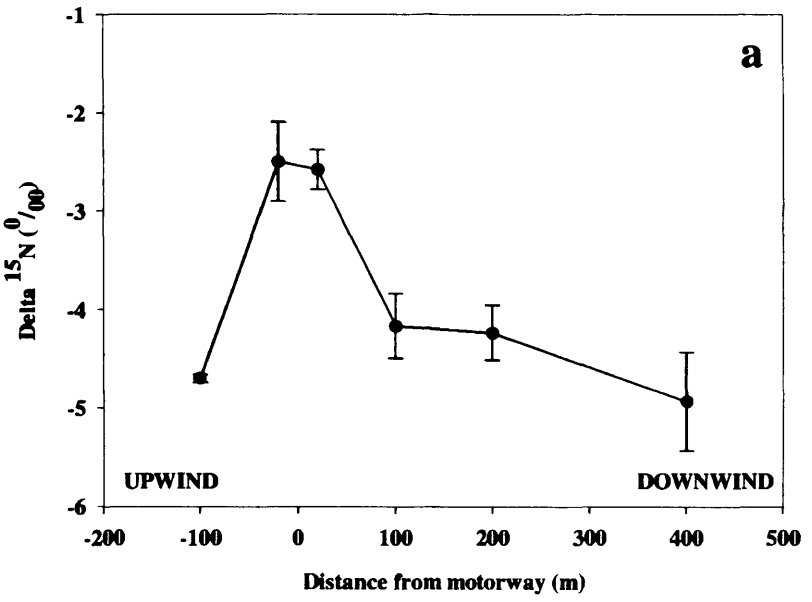
Deposition of NH_3 was measured at the site in a previous experiment (J. Pearson and A. Harrison, unpublished, CEH Merlwood UK) which reported low amounts of NH_3 deposition within the site compared to NO_2 and no differences between the motorway and the control site. It should also be noted that the deposition of NO_2 at Mardley Heath as shown in Figure 3.1. may be higher than the amount of deposition that occurs in the woodland during the growing season. This is

because diffusion tubes were set up in November when higher background NO_x concentrations occur in the UK due to the switching on of central heating systems (Goulding *et al.*, 1998).

3.2.2. $\delta^{15}N$ signature of trees

The $\delta^{15}N$ signature of leaf material was measured along the transect for two tree species, *C. monogyna* and *Quercus spp.* The lack of *S. nigra* individuals throughout the woodland interior prevented the analysis of the $\delta^{15}N$ signature of this species. The results are presented in Figure 3.2. The $\delta^{15}N$ values range from –2.6 to –7.0 $^{o}/_{oo}$ for *C. monogyna* and from –2.6 to –4.9 $^{o}/_{oo}$ for *Quercus spp.* The $\delta^{15}N$ is highest in trees adjacent to the motorway and becomes more negative as the distance along the transect increases.

The $\delta^{15}N$ of all samples taken was negative. This was most likely caused by fractionation processes in the soil and the plant. Soil N mineralisation discriminates against ^{15}N meaning that the availability of ^{15}N to plants is reduced while the $\delta^{15}N$ of the remaining SOM increases over time (Amman *et al.*, 1999). Plant uptake and assimilation also discriminate against ^{15}N however this effect is small when compared to soil mineralisation (Amman *et al.*, 1999).



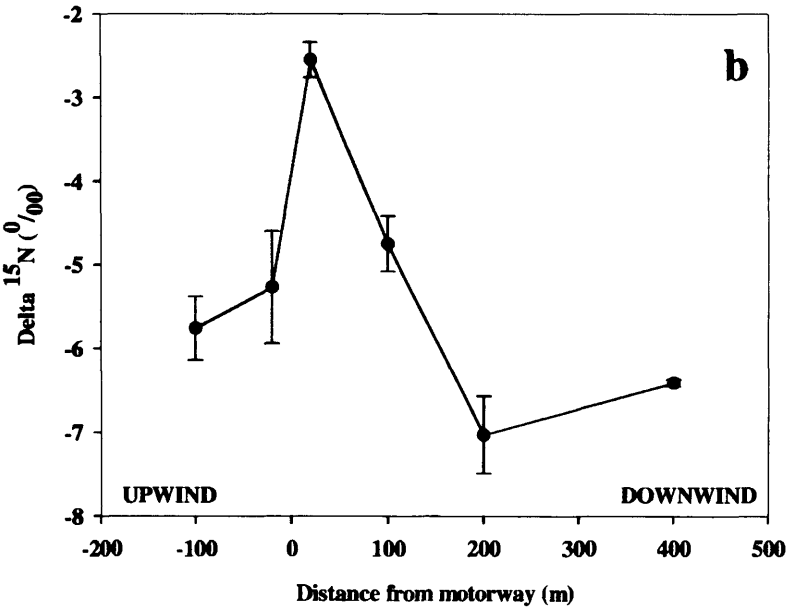


Figure 3.2. $\delta^{15}N$ signatures of leaf material collected from (a) *Quercus spp.* and (b) *C. monogyna.* Mean values ± SE error bars are shown with the sample size n= 3. Sampling took place during May 2003.

Previous work on the signature of atmospheric oxidised N has not been conclusive. According to Heaton *et al.* (1997) the $\delta^{15}N$ of atmospheric oxidised N ranges from –3 to +10 $^{o}/_{oo}$ however Amman *et al.* (1999) reported the different values of +2 to +10 and –5 to +10 $^{o}/_{oo}$ and for NO_2 and NO respectively. To complicate the matter further there are 3 forms of N deposition, namely, wet, dry and particulate, all of which may contribute different $\delta^{15}N$ signatures (Pitcairn *et al.*, 1995).

Similarly, no consistent relationship has been reported between atmospheric N pollution and plant isotopic composition (Stewart *et al.*, 2002). Inconsistencies between studies are most likely due to differences in the $\delta^{15}N$ signatures of source inputs. In many investigations (including this one) information regarding the isotopic composition of the pollutant is often based on published analyses from other studies. Unfortunately, such values are rare and highly variable and may not accurately reflect the source signature at the study site.

Nevertheless, this data shows that the motorway seems to be having a strong effect on the $\delta^{15}N$ signature of the nearby vegetation (see Figure 3.2). Since the $\delta^{15}N$ becomes less negative close to the road it seems likely that the atmospheric signature of NO_x from vehicles using the A1(M) is positive. The difference between $\delta^{15}N$ signatures at the motorway site (20 m downwind) and the control site (400 m downwind) is significant for *C. monogyna* at the 5% level ($p=0.004$). Differences between the sites were not significant for *Quercus* spp. at the 5% level ($p=0.071$).

The extent to which the $\delta^{15}N$ signature of the traffic-derived NO_x is able to influence foliar $\delta^{15}N$ signature of plants will depend on three factors, namely the strength of the source signature, how much N is taken up by the plant and the fractionation processes involved in N uptake. If traffic-derived N is taken up by plant roots following deposition to the soil, the effect on the foliar $\delta^{15}N$ signature is likely to be less than if N is taken up directly from the atmosphere. Epiphytic species such as mosses and lichens, which are dependent on atmospheric sources for supply of N avoid the complication of N uptake via the soil and hence have been recommended as suitable candidates for $\delta^{15}N$ biomonitoring (Pearson *et al*, 2000; Stewart *et al.*, 2002). The results presented in this chapter do however show that trees may also have potential as $\delta^{15}N$ biomonitoring species.

3.2.3. Total N content

The total N content of *S. nigra* and *C. monogyna* leaves are shown in Figure 3.3. *S. nigra* is a highly nitrophilous species and this is reflected by the high total N concentrations found in this study. Not only is the total N content of *C. monogyna* far lower, but it also shows less seasonal fluctuations than *S. nigra*. The total N content of ranges from 44.1 to 27.0 mg N g dwt^{-1} and from 19.7 to 16.0 mg N g dwt^{-1} for *S. nigra* and *C. monogyna* respectively.

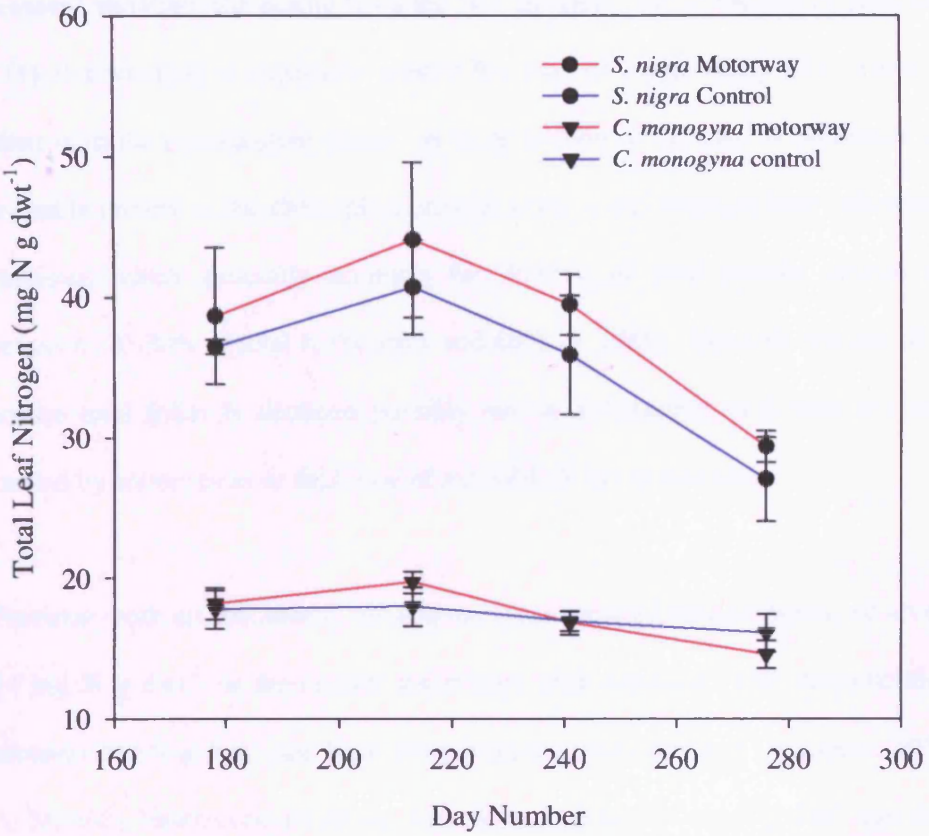


Figure 3.3. Total leaf N content of leaves of *S. nigra* (circles) and *C. monogyna* (triangles) at the motorway (red) and the control (blue) sites. Means ± SE are shown. Sampling commenced on 27/06/02.

The effect of N deposition on foliar total N has been investigated for many species. Among these are the trees investigated as part of this project, *S. nigra* and *C. monogyna* (Port and Thompson, 1980; Pitcairn *et al.*, 2002). In the Pitcairn *et al.* (2002) study, *S. nigra* foliar total N ranged from between 30 to 60 mg N g dwt^{-1} and correlated with distance from an ammonia point source (r^2=0.59). Our results for S. *nigra* are less convincing. Figure 3.3. shows that although there is a tendency for total N content to be highest in the *S. nigra* trees

adjacent to the motorway, differences were not significant. Total N shows seasonal variation, increasing from the first sampling day at the end of June (day 178) and reaching a maximum around the start of August (day 213) when the plant is in the reproductive phase. A large portion of the total N measured was probably present as the chloroplast enzyme ribulose-1,5-bisphosphate carboxylase (rubisco) which generally accounts for 30-60% of total soluble protein and between 20-30% of total N (Peoples and Dalling, 1988). Towards the end of the season total foliar N declined possibly due to a reduction in further N uptake caused by water stress or depletion of available N in the root zone.

Previous work on roadside *C. monogyna* trees revealed concentrations of around 14 mg N g dwt^{-1} in the central reservation area compared with concentrations around 6 mg N g dwt^{-1} just 10 m away from the road (Port and Thompson, 1980). At Mardley Heath concentrations range from 14.5 to 19.7 mg N g dwt^{-1} and these values are similar to the higher values presented in Port and Thompson's study. However contrary to the findings of Port and Thompson (1980), no significant differences were found between *C. monogyna* foliar N at the polluted and control sites at Mardley Heath. In field studies, the large amount of environmental variables means it can be difficult to extrapolate from one set of experiments to another. For example Port and Thompson's (1980) study site may have had a lower soil N availability than our site and it may be that the effects of traffic-derived atmospheric N on total shoot N are only be apparent where soil N supply is limiting.

3.2.4. Growth

Despite the enhanced deposition of reactive N in the modern world (Chapter 1, Section 1.3), in many habitats N is still a growth limiting factor and any increase in N availability will allow an increase in biomass production. Growth was measured in trees at the motorway and control sites and the results are shown in Figure 3.4. Both species exhibit enhanced growth at the polluted site compared to the control site. Shoot extension (Figure 3.4.a) was 93.8% and 46.3% higher at the motorway site than at the control for *S. nigra* and *C. monogyna* respectively. Two sample T-tests revealed that shoot extension was significantly different for *S. nigra* at the 1% level ($p=0.003$) and *C. monogyna* at the 5% level ($p=0.027$). The results in Figure 3.4.a strongly suggest that motorway derived N is being diverted towards extra biomass production in trees at the motorway site. The difference between the sites is larger in *S. nigra* than in *C. monogyna*, reflecting the high ability of *S. nigra* to take up and assimilate N. Number of leaf nodes (Figure 3.4.b) also appeared to be higher in trees at the motorway site although these differences were not significant.

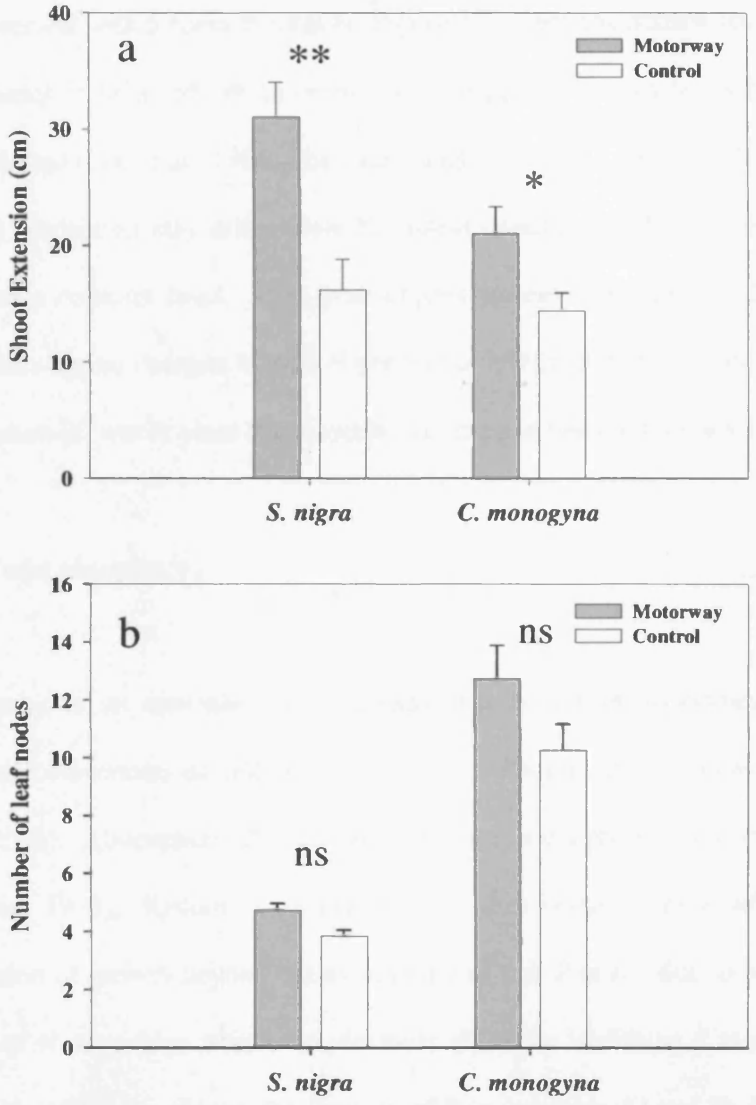


Figure 3.4. Growth measurements for *S. nigra* and *C. monogyna*. *Upper histogram* (a): Shoot extension at the motorway and the control sites. *Lower histogram* (b): Number of new leaf nodes at the motorway and the control sites. Data points are the average of 5 trees on which 15 branches were sampled. SE bars are shown. Measurements taken in September 2003. * = $p<0.05\%$; **=$p<0.01\%$, ns = not significant.

Growth in trees at the motorway site may explain why no significant differences were observed with respect to total N (Figure 3.3) since the accumulation of N in plant tissues is believed not to occur until the growth requirements for N have been satisfied (Lee *et al.*, 1986; Zhen and Leigh, 1990). In other words, increased biomass production may dilute plant N content causing total N per unit of leaf to remain at a constant level. That trees experience enhanced growth (Figure 3.4) whilst showing no changes in total N per unit of leaf (Figure 3.3) seems to suggest that dilution of whole plant N content is occurring in trees at Mardley Heath.

3.2.5. Total phosphorus

Phosphorus is an essential plant nutrient that is mainly associated with the structural components of cells such as nucleic acids and phospholipids (de Groot *et al.*, 2003). Atmospheric deposition of N can cause a reduction in total foliar P (Harrison, 1978). Reduction in total foliar P may occur as the result of (i) the stimulation of growth beyond the availability of soil P or (ii) due to acidification effects of N deposition which can decrease soil P by inhibiting P mineralization (Harrison, 1978), increasing the fixation of P to insoluble Al and Fe (Harrison *et al.*, 1988) and increasing the soil P sorption capacity (Carreira *et al.*, 1997). Since the results in Figure 3.4 showed elevated tree growth was occurring at the motorway site relative to the control it may be predicted that total foliar P will decline as the result of stimulation of growth in excess of soil available P.

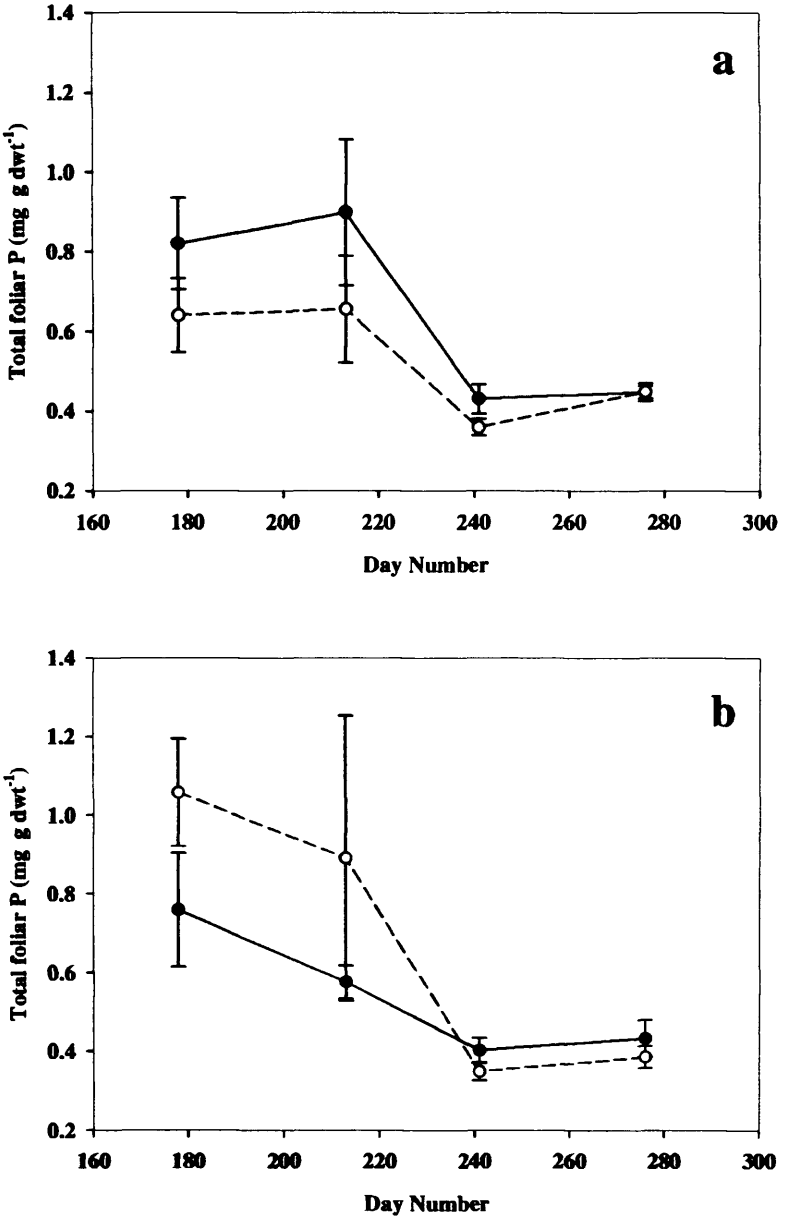


Figure 3.5. Total foliar phosphorus in (a) *S. nigra* and (b) *C. monogyna* at the motorway (solid line) and the control site (dashed line). Sampling started on 27/06/02. Data points are the average of 3 replicates. SE bars are shown.

Total foliar P concentrations in *S. nigra* are shown in Figure 3.5 (a). For *S. nigra* leaf P concentrations ranged from 0.36 to 0.90 mg P g dwt^{-1}. Concentrations appear higher at the motorway than at the control site although this difference is not statistically significant. Figure 3.5 (b) shows the total foliar P concentrations for *C. monogyna*. Foliar P Concentrations for *C. monogyna* are similar to those of *S. nigra*, ranging from 0.35 to 1.06 mg P g dwt^{-1}. Contrary to results for *S. nigra*, the concentrations of foliar P appear to be higher at the control site than at the motorway site. However, it should be noted that this difference is not statistically significant. Concentrations in both species decline towards the end of the season, most likely as the result of export of this essential nutrient for storage prior to leaf fall.

Whereas the total foliar P concentrations are quite similar between both species sampled, the total N concentrations showed much more variation between these two species (Figure 3.3.). As a result the foliar N:P ratios of these two species vary greatly (Table 3.1).

Table 3.1 Foliar N:P ratios in *S. nigra* and *C. monogyna* sampled in 2002

Day Number	***Foliar N:P ratios***			
	S. nigra		*C. monogyna*	
	Motorway	Control	Motorway	Control
178	47.1	57.0	23.9	16.8
213	49.0	62.1	34.1	30.2
241	82.7	97.3	41.6	48.3
276	88.2	80.0	40.3	44.8

Several workers have proposed the use of foliar N:P ratios as an alternative for using total N as a biomarker (Zinke, 1980; Mohren *et al.*, 1986; Ericsson *et al.*, 1993).

For instance, following work on *Picea abies,* Ericsson *et al.* (1993) reported that an N:P ratio of 12.5-14 corresponded to N deposition effects. Similarly, Tessier and Raynal (2003) used thresholds of <14 for N limitation and >16 for P limitation to demonstrate that under story vegetation at Catskill Mountains was N saturated. In the UK, Kirkham (2001) found that N:P ratios of *Nardus stricta* correlated with N deposition and suggested that upland *Calluna* dominated regions are undergoing conversion from N limited to P-limited ecosystems.

The foliar N:P ratios presented in Table 3.1 are much higher than those quoted in the literature, perhaps because this work uses more nitrophilous species. It seems unlikely therefore that a single N:P threshold can be used for all species as a biomarker for N deposition. Seasonal changes appear to be large due to export of both of these nutrients at senescence, therefore if N:P ratios are used as biomarkers the time of year at which sampling takes place should be standardised.

The use of N:P ratios as biomarkers is based on the idea that with increased N deposition the N content of the plant will increase relative to other nutrients such as P. However, as Figure 3.3 and Figure 3.5 show, there were no significant effects of N deposition on either total N or total P and this may explain why the data in Table 3.1 do not show any consistent trends which may have indicated that they are a useful biomonitoring resource.

3.2.6. Conclusions

The data shows significantly higher amounts of deposition at the motorway site relative to the control. Deposition within the woodland ranged from 14.8 to 40.7 Kg N ha^{-1} yr^{-1}. These values are in excess of the current critical loads set for UK woodlands thus indicating that there is the potential for N to be causing damage to under story vegetation throughout this site. The marked decline in N deposition along the transect, away from the motorway, confirms the hypothesis that the motorway is acting as a point source of atmospheric NO_x. This gradient in pollution exposure along the transect is assumed to be a major factor when considering vegetation response along the transect.

Traffic-derived N appears to be contributing to the N nutrition of the plants at the motorway site since the ^{15}N data shows elevated $\delta^{15}N$ values relative to the control. Whether this N is entering the plant directly through the stomata or following deposition to the soil and uptake via the roots will be considered in later chapters. It appears that both *S. nigra* and *C. monogyna* may benefit from traffic-derived N deposition at the motorway site by increasing growth since branch elongation was significantly higher in both of these species at this site relative to the control. According to Nilsson (1986), a positive growth response to N additions indicates that the habitat is not N saturated. Therefore since both *C. monogyna* and the more nitrophilous *S. nigra* demonstrate this growth response it can be assumed that Mardley Heath is not N saturated.

At the same time, it also appears that Mardley Heath is not nutrient limited since additions of N are only thought to stimulate growth when other nutrients are not limiting. Indeed total leaf P concentrations did not show any significant differences between the sites. Contrary to several other studies that have shown total N to be significantly affected by enhanced N deposition, the results presented in this chapter do not show any significant effects of N deposition on total N content. This may be because while the N content of the whole plant is elevated at the motorway site, total N per unit of leaf may remain fairly constant due to dilution by enhanced growth. The same may be true of total P which also may have been diluted by enhanced growth. This agrees with the finding of Schulze (1989) that trees respond to additions of N by increasing leaf biomass at a rate that holds the concentration of shoot N at a nearly constant level.

It can be concluded that in habitats such as this one where supply of nutrients does not restrict plant growth, foliar N, P and N:P ratios are not useful biomarkers of N deposition. It seems instead that $\delta^{15}N$ signatures and simple growth measurements may provide a better indication of the effect of N deposition on roadside vegetation. Although the ^{15}N content of leaves is likely to be diluted by growth in a similar way to total N, the effect of motorway derived N still appears to be apparent.

CHAPTER 4

EFFECTS OF NITROGEN DEPOSITION ON TREE PHYSIOLOGY

4.1. Introduction

4.1.1. Atmospheric uptake of nitrogen by plants

Where trees are exposed to atmospheric NO_x, this may be absorbed by the foliage. Uptake of NO_x occurs predominantly via the stomata (Hanson and Lindberg, 1991). Deposition of NO_2 to trees is relatively simple to calculate as it based entirely on air NO_2 concentrations and stomatal conductance (Fowler, 2002). NO release from nitrification and denitrification in soils also contributes to foliar uptake as NO reacts with O_3 to give NO_2 (Fowler, 2002). The uptake of NO_2 has the effect of generating a net canopy compensation point above which there is a net NO_2 emission from the canopy (Fowler, 2002).

Foliar entry may circumvent a plant's regulatory control over root N uptake (Norby, 1989; Stulen *et al.*, 1998). Once inside, NO_2 dissolves in the apoplast to yield HNO_2 and HNO_3 which in turn dissociate to give NO_3^-, NO_2^- and H^+ all of which are able to permeate membranes but only two of which, NO_3^- and H^+, are commonly present within cells (Welburn, 1990). It is supposed that plants can deal with such inputs by a combination of mechanisms, namely the inhibition of

root uptake of N, storage of excess NO_3^- in the vacuole, release of NO_x from the leaves and assimilation into proteins (Nishimura *et al.*, 1986; Welburn, 1990; Soares *et al.* 1995).

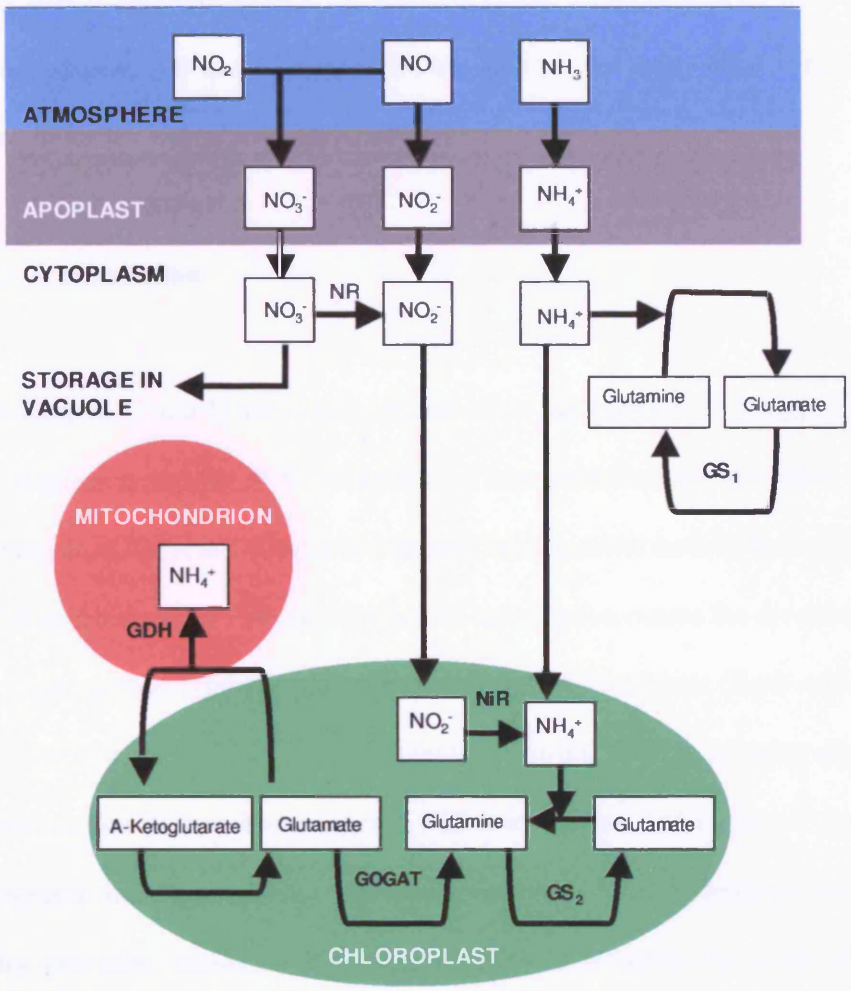


Figure 4.1. Main biochemical processes involved in the foliar uptake and assimilation of atmospheric NH_3 and NO_x. NR represents nitrate reductase, NiR represents nitrite reductase, GDH represents glutamate dehydrogenase, GOGAT represents glutamate synthase, GS1 and GS2 represent cytosolic and chloroplastic isoforms of glutamine synthetase respectively (adapted from Lea *et al.*, 1994).

4.1.2. Perturbation of metabolic processes

Since the uptake of foliar N bypasses the plant's regulatory control over uptake (Norby, 1989), exposure to NO_x can have important consequences for not only the uptake, transport and assimilation of N, but also for the many other biochemical processes that are linked with N metabolism.

4.1.2.1. C Metabolism

Metabolism of C and N are closely related since assimilation of NH_3 into amino acids requires a supply of C skeletons. These skeletons are provided by the tricarboxylic acid cycle (TCA) and removed intermediates have to be replenished by increased activity of PEP carboxylase. N assimilation causes the diversion of C away from sucrose synthesis and towards amino acid synthesis (Raab and Terry, 1995). NO_3^- itself may act as a signal molecule in this process bringing about the phosphorylation of two key enzymes, PEP carboxylase and sucrose-P synthase (Champigny and Foyer, 1992). Phosphorylation of PEP carboxylase increases activity and thus results in the replenishment of tricarboxylic acids that are necessary for N assimilation. Conversely, phosphorylation of sucrose-P synthase reduces activity of this enzyme thereby bringing about a reduction in sucrose synthesis (Champigny and Foyer, 1992).

Raab and Terry (1995) examined the effects of N pollution on the availability of non-structural carbohydrates. In their experiment N deposition caused the depletion of foliar starch, sucrose, maltose and glucose while stimulating

glutamine production. It was also shown that excess N was able to increase the activities of some glycolytic/krebs cycle enzymes. Similarly, Grulke *et al.* (2001) discovered that the carbohydrate concentration of pine needles and the fine root monosaccharide concentrations were decreased in *Pinus ponderosa* at the most heavily polluted end of a pollution gradient.

4.1.2.2. Amino Acids

Increases in the amount of certain amino acids as the result of exposure to N deposition have been reported (VanDijk and Roelofs, 1988; Ericsson *et al.*, 1993; Hun and Schulz, 1996; Calanni *et al.*, 1999; Pitcairn *et al.*, 2003). Proline, glutamic acid, aspartic acid and glutamine tissue pools have all been shown to increase in response to excess N deposition by these authors. However, the amino acid that is most frequently linked with N deposition is arginine. This is the only amino acid to have four N atoms within its structure and is considered to be the primary excess N binding substance (Calanni *et al.*, 1999). Calanni *et al.* (1994) showed that levels of this amino acid in *Picea englemanni* needles were highly variable and corresponded to diurnal variations in atmospheric N deposition after a 4 (±2) hour time delay.

4.1.2.3. N transport in the xylem

Plants have the capacity to adjust root N uptake to meet the demands of the shoots. In an experiment conducted by Siebrect and Tischner (1999) using poplar (*P. tremula* x *P. alba*), it was shown that when the NO_3^- content of the rooting

medium was increased above an optimum level this had no effect on the N status of the xylem exudates. This experiment demonstrates that this species has control over NO_3^- uptake and will not take up excessive amounts of this nutrient. Similarly Clement, Hopper and Jones (1978) stated that external soil NO_3^- concentrations above an optimal level have no effect on the relative growth rate. Plant growth requires not only N but a range of other mineral nutrients and therefore the amount of NO_3^- which constitutes this optimal level will largely depend on the availability of other essential nutrients. If atmospheric N is taken up via the leaves this may satisfy a proportion of the shoot's N demands thus bringing about the down-regulation of root uptake. This idea is supported by the work of Nasholm (1998) who revealed low root uptake of NH_4^+ and NO_3^- in forest stands subject to high loads of N deposition.

Regulation of N uptake may occur by means of feedback mechanisms. For example shoots may export specific amino acids to the roots via the phloem in order to signal a change in shoot N demands (Muller and Touraine, 1992; Gessler *et al.*, 1998a; Gessler *et al.*, 1998b). Other likely candidates as N signalling molecules include malate and carbohydrates which may signal the C:N status of the shoot (Touraine *et al.*, 1992; Rideout *et al.*, 1994; Soares *et al.*, 1995; Pearson and Soares, 1998).

4.1.2.4. Nutrient status

Aside from being an important N transport molecule, NO_3^- also serves to balance charge equivalents within the xylem (Marschner, 1995). Decreased amounts of

NO_3^- in the xylem as the result of atmospheric N inputs could mean that malate may be required as a counter ion in place of NO_3^- (Ben-Zioni *et al.*, 1971; Pearson and Soares, 1998). Given that NO_3^- uptake is closely related to uptake of other ions, a decrease in NO_3^- uptake may be accompanied by lower uptake of many other essential ions including the cations, K^+, Ca^{2+}, Mg^{2+}, Na^+ and the anions SO_4^{2-}, PO_4^{3-} and Cl^- (Gniazdowska *et al.*, 1998; Siebrecht *et al.*, 1999). Thus, exposure to N pollution can bring about symptoms characteristic of nutrient deficiencies. Nutrient deficiencies may also occur where additional N stimulates growth in excess of the supply of other soil nutrients. This situation may be exacerbated by low nutrient availability in N polluted soils due to soil acidification and leaching of NO_3^- which both lead to a reduction in soil nutrients (Schulze, 1989).

4.1.2.5. Acid/base balance

Atmospheric uptake of N can have severe effects on the acid-base regulation of the plant (Raven, 1988). The effects are different for NH_3 and NO_x since the dissolution of NH_3 in the apoplast it increases the pH whereas dissolution of NO_x lowers the pH. Subsequent metabolism of these has the opposite effect with NO_x assimilation producing basic OH^- ions and NH_3 assimilation producing acidic H^+ ions. Thus assimilation of NH_4^+ usually occurs in roots where excess H^+ can be disposed of by efflux to the soil (Raven, 1986). Shoot assimilation does not allow H^+ to be disposed of in this way. It has been suggested that H^+ ions resulting from atmospheric NH_3 uptake are excreted into the apoplast thereby lowering the pH. Plants with higher capacities for foliar NO_3^- assimilation may be better equipped

to deal with shoot H^+ since they generate more OH^-, increasing H^+ buffering capacity. Such plants may be more resistant to damage caused by atmospheric pollution (Pearson and Soares, 1995). Pearson and Soares (1995) found a positive correlation between leaf NR activity and foliar buffering capacity.

4.1.2.6. Seasonal Shifts

In natural systems, N availability typically varies throughout the year (Runge, 1983). Generally plants adapt to this variable N supply by storing N during periods of ample availability for use during periods of intensive growth. In fact, most species show seasonal fluctuation in N storage compounds with high amounts during spring and autumn and low amounts in early summer. N deposition can alter the periodicity of N supply and therefore may alter the normal pattern of seasonal fluctuation so that high levels of storage compounds occur during periods of intensive growth (Nordin and Nasholm, 1997).

4.1.3. Aims and Objectives

This chapter aimed to partly fulfil the second objective of this thesis by investigating the effects of increased N deposition on tree physiology. Work was conducted on trees at the Mardley Heath field site. Tissue extract and xylem sap was analysed for a range of metabolites including NO_3^-, amino acids, NH_4^+, soluble sugars and PO_4^{3-} on a monthly basis during 2001 and 2002. From this work it was hoped to achieve a greater understanding of the effects of NO_x deposition on sugar assimilation and PO_4^{3-} transport, tissue NO_3^- content and N

transport in the xylem. Sampling is carried out throughout the year in order to detect seasonal changes that occur with respect to these metabolites.

4.2. Results and Discussion

4.2.1. Notes on data interpretation

It is important that the reader bears in mind the following facts when considering the results presented in this Chapter. Firstly, the tissue concentrations quoted herein represent concentrations in the whole leaf tissue and hence provide no information with respect to cytoplasmic and vacuolar concentrations. Secondly, xylem sap was sometimes difficult to obtain particularly from *C. monogyna* branches and so on some sampling occasions only a few µl could be collected thus allowing only a few biochemical analyses to be performed. Therefore, some of the data showing xylem sap analyses is either absent or is based upon only one or two replicates. Where this is the case however, it is noted in the figure legend. One last thing to note is that xylem sap concentrations may not represent actual flux to the leaf since transpiration flow and the supply to individual leaves have not been measured.

4.2.2. Nitrate concentrations in tissue and xylem sap

Tissue and xylem concentrations of NO_3^- in *S. nigra* plants during 2002 are shown in Figure 4.2. Concentrations in 2001 were measured and found to show a similar

trend. Levels peak around day 100 (mid April) when concentrations at the motorway site were 2.73 mg NO_3^- g fwt^{-1} and 0.23 mg NO_3^- ml^{-1} in tissue and xylem sap respectively. High amounts of NO_3^- around this time of year may be due to the high N requirement of developing leaves for growth (Gonzales-Real and Bailler, 2000). Spring increases in NO_3^- at this time of the year is likely to be provided from N mobilisation from other organs or enhanced uptake from the soil. Levels of soil nutrients are high in the early spring due to increased microbial activity around late winter or early spring (Fitter and Hay, 1991).

These early seasonal flushes in tissue and xylem NO_3^- are followed by a sharp decline in concentrations. Tissue NO_3^-concentrations then proceed to increase steadily for the remainder of the growing season. However, the increase in tissue NO_3^- concentrations does not seem to be due to increased supply from the xylem, which remains fairly constant for the remainder of the season. Possible explanations for this increase are the accumulation of stored NO_3^- in the cell vacuole throughout the growing season (Gransted and Huffaker, 1982).

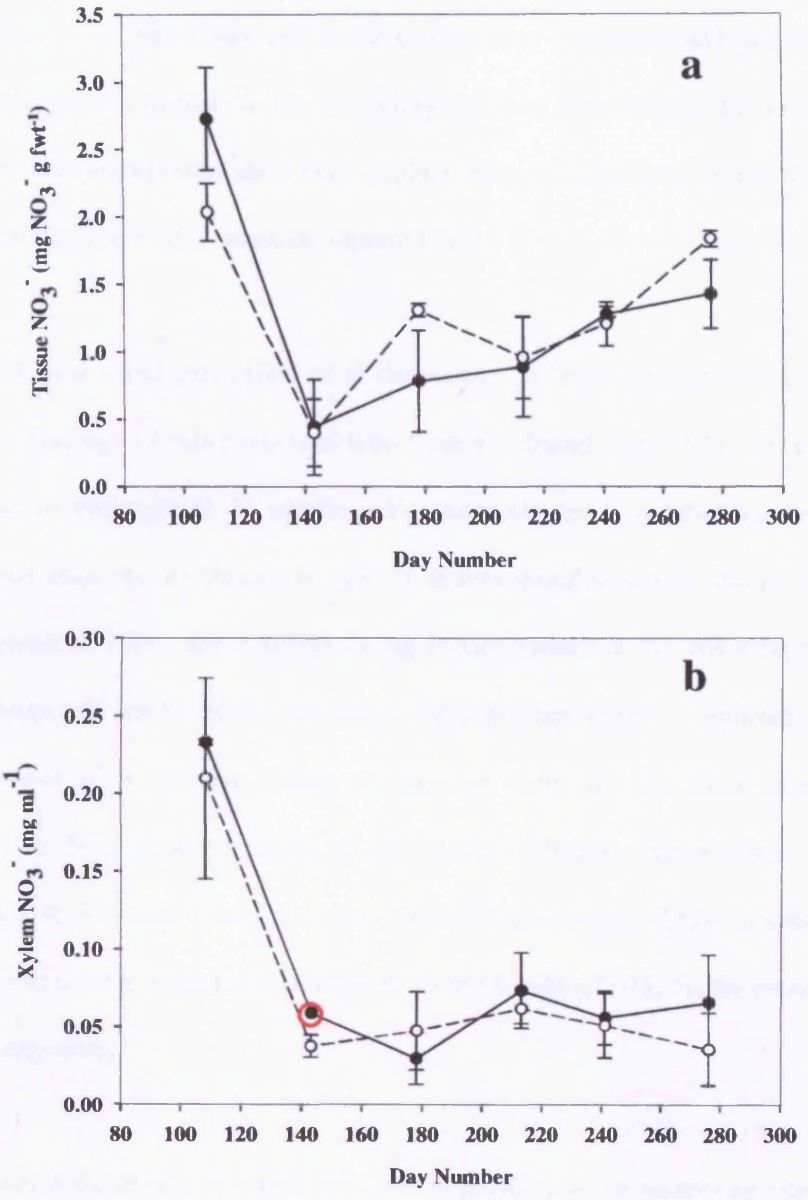


Figure 4.2. Shoot *S. nigra* NO_3^- concentrations in (a) leaf tissue and (b) xylem sap at the motorway (solid line) and the control site (dashed line) for 2002. Sampling commenced on 18/04/02 and ended on 03/10/02. Data points are the average of 3 replicates unless marked with a red circle, in which case only one replicate was obtained. SE bars are shown.

There are no significant differences between the tissue NO_3^- concentrations in *S. nigra* trees at the motorway and at the control site. As mentioned in Chapter 3, stimulated growth of trees at the motorway site may have diluted the amounts of foliar N compounds and thus may explain why no significant differences are found for the tissue NO_3^- amounts reported here.

This lack of a significant effect of N deposition on NO_3^- concentrations concurs with the findings of other workers who have not found tissue NO_3^- increases in response to atmospheric N uptake. For example using a labelling study on *Phaseolus vulgaris*, Rogers *et al.* (1979) demonstrated that only 4% of foliar N was present as NO_3^-, the majority being incorporated via the NR enzyme into proteins/nucleic acids (63%) or amino acids/amides (33%). Similarly, field observations of N polluted stands of *Quercus robur* did not show substantial changes in NO_3^- concentrations in the leaves (Thomas and Buttner, 1998). According to Srivastava and Ormond (1989) changes in tissue NO_3^- concentration in response to NO_x exposure will depend on the supply of NO_3^- to the roots at the time of exposure.

In Chapter 3 the results revealed higher N deposition at the motorway site along with stimulated growth and a change in $\delta^{15}N$ signature which points towards the possibility of foliar uptake of traffic-derived N. Since plants are able to regulate root N uptake in accordance with shoot demands it may be predicted that if atmospheric deposition is being taken up by the foliage and acting as an additional N source, root N uptake will be down-regulated (Gessler *et al.*, 1998b; Siebrect

and Tischner, 1999). The results in Figure 4.2.b however show that xylem concentrations of NO_3^- are unaffected by proximity to the motorway. Nevertheless it should be noted that other N-containing metabolites can also transport N in the xylem and therefore while changes in NO_3^- concentrations have not been detected, the motorway may affect root-shoot transport of N via changes in other metabolites or through differences in xylem flow rates which were not measured in this study.

Concentrations of xylem and tissue NO_3^- in *C. monogyna* plants are shown in Figure 4.3. The xylem NO_3^- concentrations are very low perhaps suggesting that NO_3^- is not an important xylem N transport molecule for this species. Levels of tissue NO_3^- are also low with the exception of early in the growing season when levels are 1.11 and 1.25 mg NO_3^- g fwt^{-1} at the motorway and the control sites respectively. Since NO_3^- concentrations in the xylem are very low it is unlikely that this early peak in tissue NO_3^- is provided via the xylem, that is unless a large influx occurred prior to the first sampling date. Other potential sources of early seasonal tissue NO_3^- include NO_3^- stored in buds over the winter or NO_3^- inputs via the phloem. When leaves are young they experience a phloem importing stage during which N that had been stored over winter is translocated to leaves (Marschner, 1995). The xylem becomes more important for supplying N when the leaves are fully expanded and the transpiration stream is well established (Marschner, 1995).

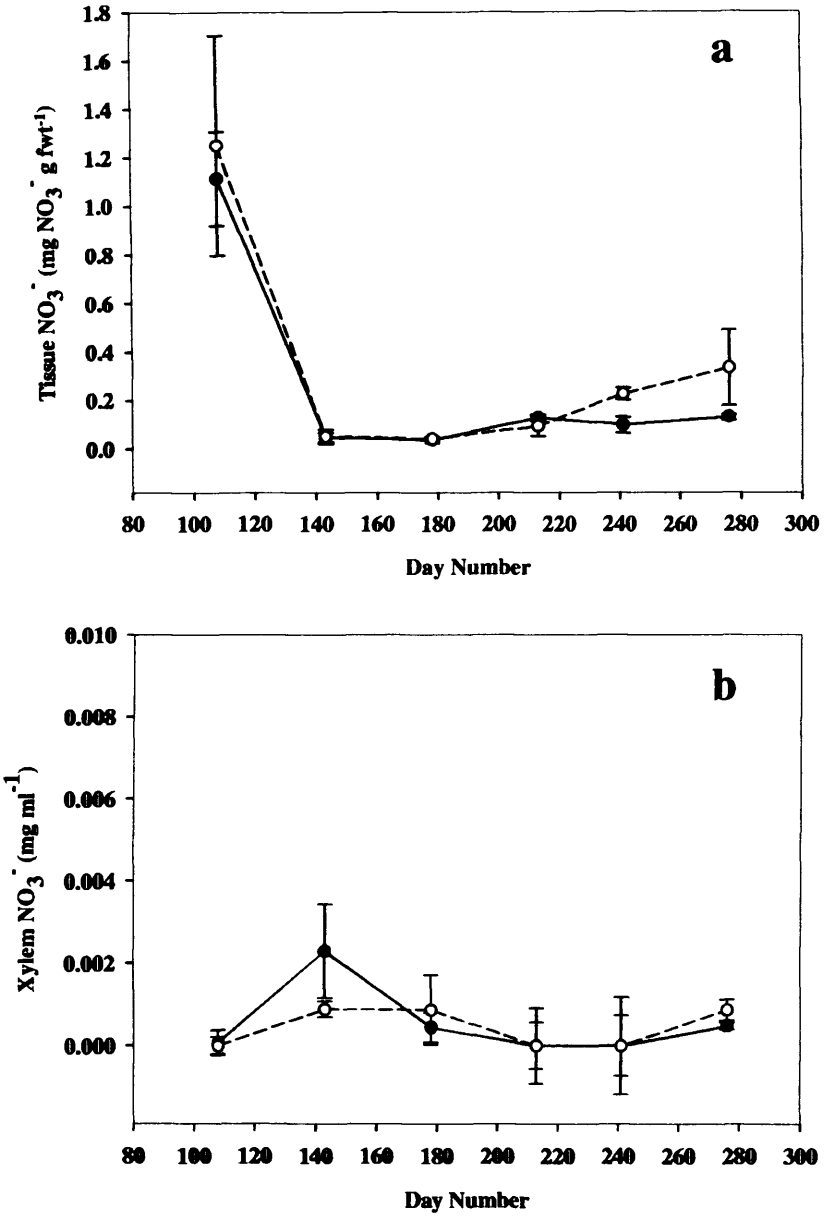


Figure 4.3. Shoot *C. monogyna* NO_3^- concentrations in (a) leaf tissue and (b) xylem sap at the motorway (solid line) and the control site (dashed line) for 2002. Sampling commenced on 18/04/02 and ended on 03/10/02. Data points are the average of 3 replicates. SE bars are shown.

It seems unlikely that N deposition will affect xylem NO_3^- concentrations as amounts are low suggesting that this is not an important xylem N transport molecule for *C. monogyna*. Indeed the results show little difference between xylem NO_3^- concentrations at the motorway and the control sites. Tissue concentrations also show no significant differences between the motorway and the control sites. However earlier results (3.2.2) did show the influence of traffic-derived N on the δ-^{15}N signature and therefore the contribution of traffic-derived N to the N budget of this species. As discussed earlier this may be due to the incorporation of atmospheric N into amino acids and proteins rather than cellular NO_3^- (Rogers *et al.*, 1979), the dilution of N compounds by growth or alternatively may point towards deposition of traffic NO_x to soil followed by root uptake and assimilation before transfer to the shoots as amino acids.

4.2.3. Other xylem N transport molecules – amino acids and ammonium

The major N transport molecules in the xylem are ureides, amino acids and nitrate (Marschner, 1995; Parsons and Sunley, 2001).

4.2.3.1 Xylem ammonium

Seasonal data for NH_4^+ concentrations in the xylem sap of *S. nigra* and *C. monogyna* are shown in Figure 4.4. Concentrations are similar for both species ranging from 0 to 8.13 and 0 to 5.9 $\mu g\ ml^{-1}$ for *S. nigra* and *C. monogyna* respectively.

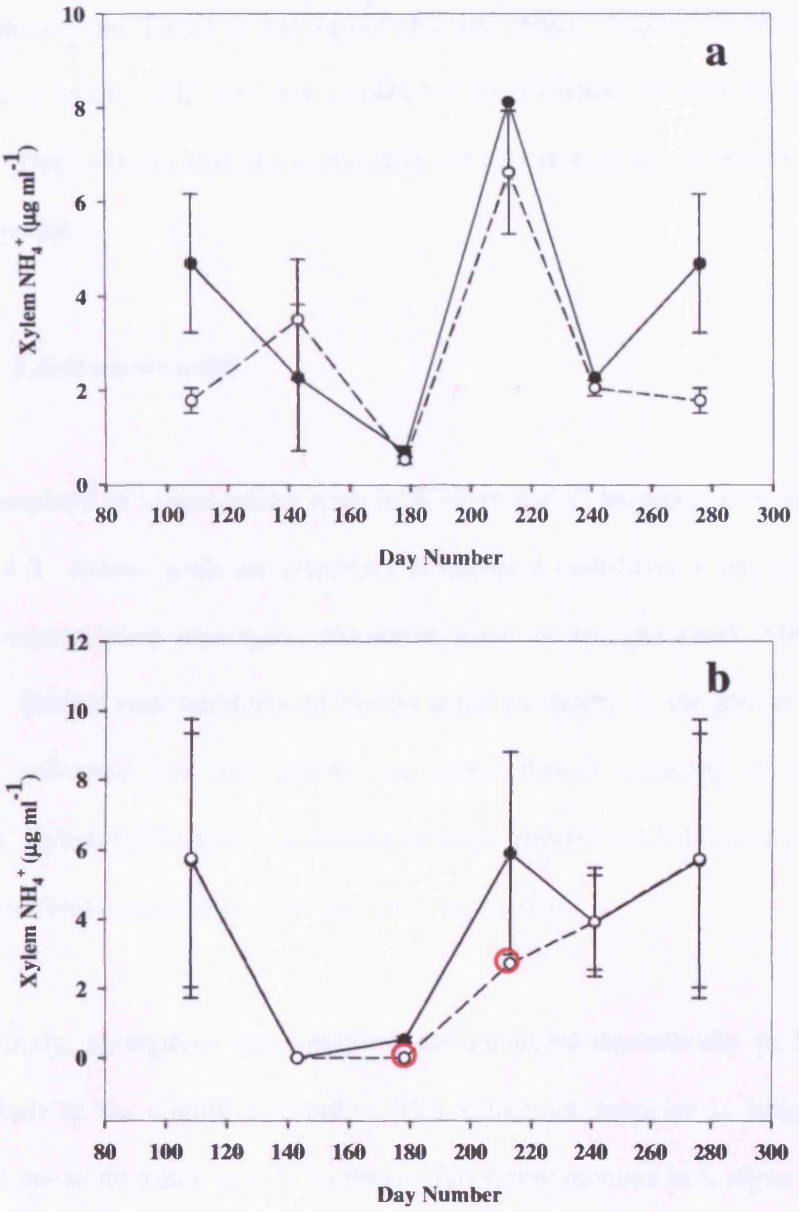


Figure 4.4. NH_4^+ concentrations in xylem sap of (a) *S. nigra* and (b) *C. monogyna* at the motorway (solid line) and the control site (dashed line) for 2002. Sampling commenced on 18/04/02 and ended on 03/10/02. Data points are the average of 3 replicates unless marked with a red circle, in which case only one replicate was obtained. SE bars are shown.

Since NH_4^+ is toxic at low concentrations and tends to be assimilated in the roots it is generally not found in the xylem (Raven, 1986). Figure 4.4 reveals low amounts of xylem NH_4^+ for both species and no consistent trend in the seasonal profile. This indicates that this molecule is not important in xylem N transport for either species.

4.2.3.2. Xylem amino acids

Concentrations of xylem amino acids in *S. nigra* and *C. monogyna* are shown in Figure 4.5. Amino acids are important N transport molecules in the xylem, the most common being asparagine, glutamine, aspartate and glutamate (Marschner, 1995). Similar concentrations of amino acids are found in the xylem of both species indicating that root uptake and assimilation is occurring in both tree species. Xylem amino acid concentrations range from 0.71 to 9.14 μmol ml^{-1} in *S. nigra* and from 0.00 to 3.41 μmol ml^{-1} in *C. monogyna.*

Interestingly, springtime concentrations are increased dramatically in *S. nigra* individuals at the control site only. This difference however is insignificant perhaps due to the small sample numbers. The spring increase in *S. nigra* is likely to be due to the greater availability of soil nutrients in combination with enhanced N demands from shoots. What is surprising however is the fact that concentrations at the motorway site do not show this springtime flush. It is proposed that xylem flux to the shoots is reduced at the motorway site because a proportion of the *S. nigra*'s shoot N requirements are satisfied by foliar uptake of atmospheric traffic-derived NO_x.

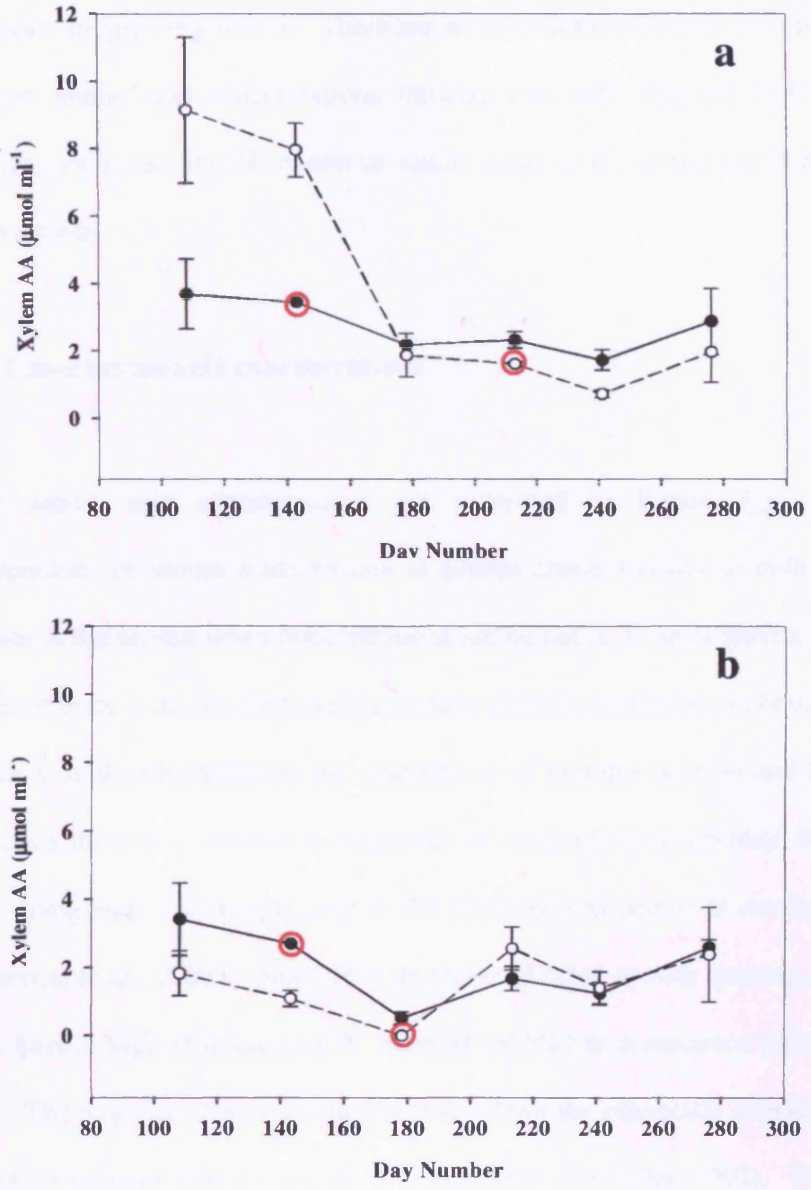


Figure 4.5. Amino acid concentrations in xylem sap of (a) *S. nigra* and (b) *C. monogyna* at the motorway (solid line) and the control site (dashed line) in 2002. Sampling commenced on 18/04/02 and ended on 03/10/02. Data points are the average of 3 replicates unless marked with a red circle, in which case only one replicate was obtained. SE bars are shown.

Concentrations of amino acids in *C. monogyna* show no seasonal trends, indicative of a constant supply of amino acids to the shoots by the xylem throughout the growing season. There are no significant effects of the motorway on xylem amino acid concentrations showing that traffic-derived N is neither inhibiting nor enhancing N uptake as amino acids in *C. monogyna* trees at the motorway site.

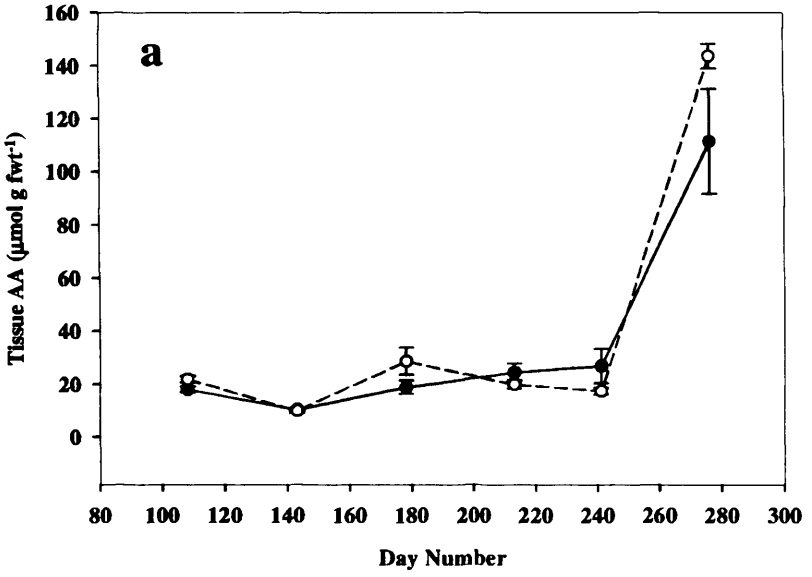
4.2.4. Tissue amino acid concentrations

Tissue amino acid concentrations are presented in Figure 4.6. Tissue concentrations of amino acids remain at similar concentrations in both species until late in the season when concentrations are higher in *S. nigra* leaves. Late in the season there is an increase in tissue amino acid concentrations in both species and this is probably caused by the dismantling of proteins prior to leaf fall. At senescence there is a massive mobilization of nutrients from obsolete leaves to newly developing and storage parts of the plant such as perennial woody tissues (Temperton *et al.*, 2003). Since N is an essential often growth limiting element leaves have a high efficiency of N removal (>65%) at senescence (Marschner, 1995). The majority of redistributed N arises from the enzymatic degradation of chloroplast proteins such as rubisco (Hörtensteiner and Feller, 2002). These are broken down by peptide hydrolases into amino acids which, following the metabolic interconversion of breakdown products and are eventually exported from the leaf via the phloem (Hörtensteiner and Feller, 2002).

High autumnal amino acid concentrations in *S. nigra* relative to *C .mongyna* can be attributed to the relatively high levels of total N in this species (Section 3.2.3). Total N concentrations in *S. nigra* were seen to decline towards the end of the growing season (Figure 3.3). This decline is probably due to degradation and export of N and can be related to the high levels of amino acids that are observed in Figure 4.6.a. The amino acids that are most likely to be responsible for this rise are glutamine and asparagine which are common mobile forms of N (Marschner, 1995).

According to the literature, N deposition causes increases in several amino acids, in particular N-rich arginine (VanDijk and Roelofs, 1988; Ericsson *et al.*, 1993; Hun and Schulz, 1996; Calanni *et al.*, 1999; Pitcairn *et al.*, 2003). Differences between the motorway and the control site however are not apparent in *S. nigra* data (Figure 4.6.a). However, it should be remembered that data presented here is a measurement of the total pool of amino acids and therefore changes in the relative abundance of specific amino acids may be occurring undetected.

In *C. monogyna* however there appear to be differences between the two sites with respect to tissue amino acids concentrations. From May onwards the tissue concentrations in individuals at the control site are seen to increase at a higher rate than those at the motorway site. This difference was shown to be significant from day 179 onwards at the 5% level ($p= 0.034$).



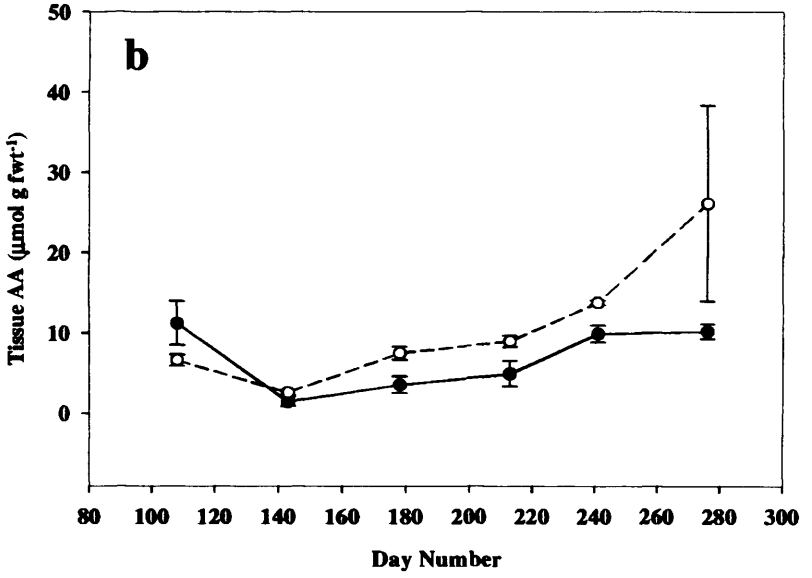


Figure 4.6. Amino acid concentrations in leaf tissue of (a) *S. nigra* and (b) *C. monogyna* at the motorway (solid line) and the control site (dashed line) in 2002. Sampling commenced on 18/04/02 and ended on 03/10/02. Data points are the average of 3. SE bars are shown Note the very different y-axis scales on these two graphs.

As mentioned previously, increases in tissue amino acid concentrations are often associated with preparation for senescence as cellular proteins are broken down into their constituent components in preparation for export from the leaf prior to leaf fall. Thus, increases in amino acids at the control site occurring earlier in the year than at the control site may indicate perhaps that senescence is somehow being delayed at the motorway site. Although senescence operates under the active control of genes it is modified by environmental signals (Gan and Amasino, 1997). Environmental differences between the sites such as supply of N or other factors such as CO_2 concentrations or temperature may have affected the onset of senescence so that the associated physiological changes occur at a later date at the motorway site. Indeed N deposition has been shown to increase concentrations of the plant hormone, cytokinin (Collier *et al.*, 2003), which plays an important role in delaying leaf senescence. Cytokinins are rapidly metabolized by cells (Noodén and Letham, 1986) and a continuous supply is necessary to prevent the onset of senescence (Marschner, 1995). Increased N deposition at the motorway site may therefore be delaying senescence in *C. monogyna* trees at this site.

4.2.5. Tissue Sugar Concentrations

Enhanced N assimilation occurring as the result of N deposition has previously been shown to decrease sugar concentrations (Raab and Terry, 1995; Grulke *et al.*, 2001). The effect of N deposition on sugar content is not apparent in this study since no significant differences were detected between the two sites (Figure 4.7). There is however marked seasonal variation in the sugar concentrations over the year. *S. nigra* shows a decline in concentrations following an initial mid-April

peak of 32.10 mg sugar g fwt^{-1} (motorway) and 31.34 mg sugar g fwt^{-1} (control). This decline is most likely due to a reduction in photosynthesis. Diminished sugar concentrations as the result of a decreased photosynthesis lead to the activation of thylakoid breakdown in preparation for senescence (Buchanan-Wollaston, 1997). Thylakoid membranes consist mainly of lipids which are converted into mobile sugars through the glyoxylate cycle. This may coincide with the second peak in tissue sugar concentrations in *S. nigra* leaves. By the final sampling day on 03/10/02 sugar concentrations have dropped further most likely because the export of readily mobile sugars from the leaves at senescence has already begun to occur.

In *C. monogyna* sugar concentrations remain fairly constant at the start of the year before rising, most likely as the result of lipid degradation, and then declining sharply prior to leaf drop. Although not significantly different, it appears that concentrations fall more sharply at the control site compared with the motorway site which fits with the idea mentioned previously of delayed senescence occurring at the motorway site.

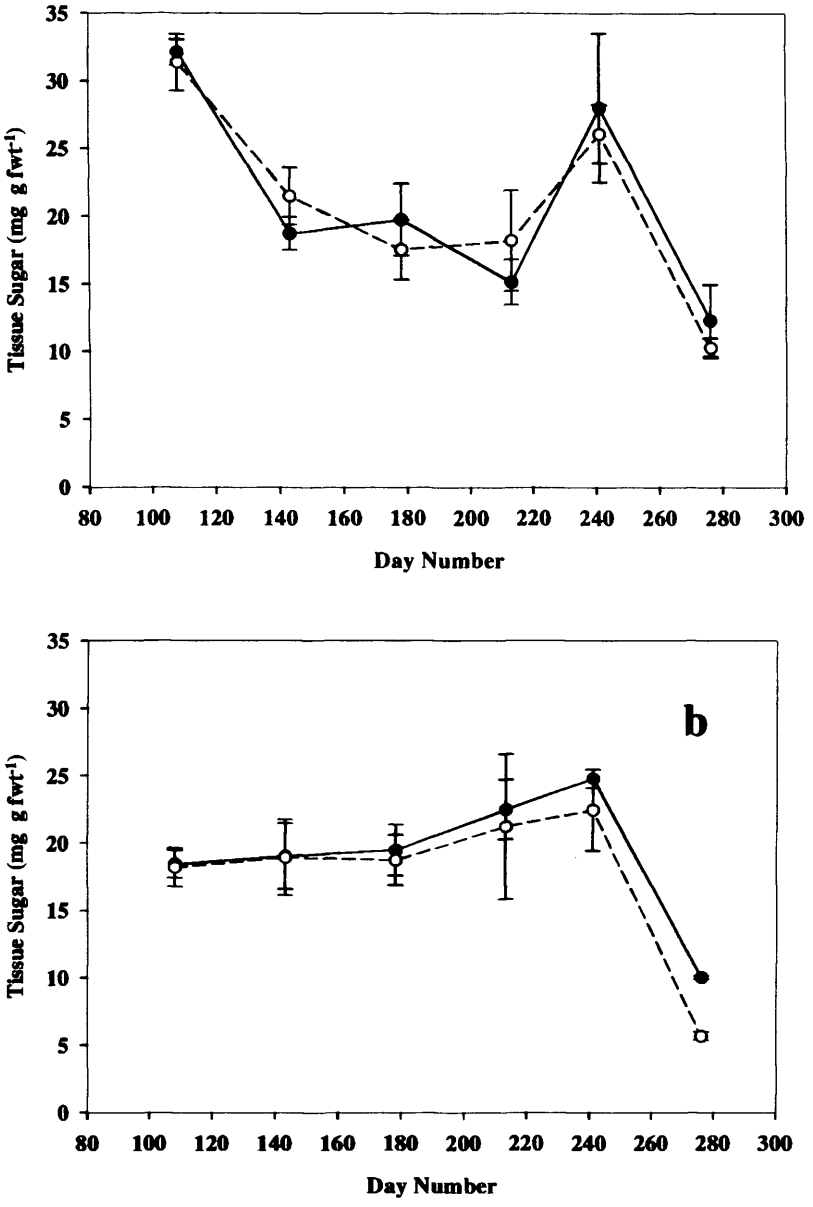


Figure 4.7. Soluble sugar concentrations in leaf tissue of (a) *S. nigra* and (b) *C. monogyna* at the motorway (solid line) and the control site (dashed line) in 2002. Sampling commenced on 18/04/02 and ended on 03/10/02. Data points are the average of 3 replicates. SE bars are shown.

4.2.6. Xylem phosphate concentrations

Xylem PO_4^{3-} concentrations for *S. nigra* are shown in Figure 4.8. Insufficient sap collection for *C. monogyna* meant that analysis of xylem PO_4^{3-} amounts was not performed for this species. Concentrations in *S. nigra* are highly variable throughout the year and may be related to day-to-day changes in PO_4^{3-} uptake rather than a seasonal trend however amounts do appear to be slightly higher towards the start of the season, perhaps corresponding to high nutrient requirement of newly formed, developing leaves.

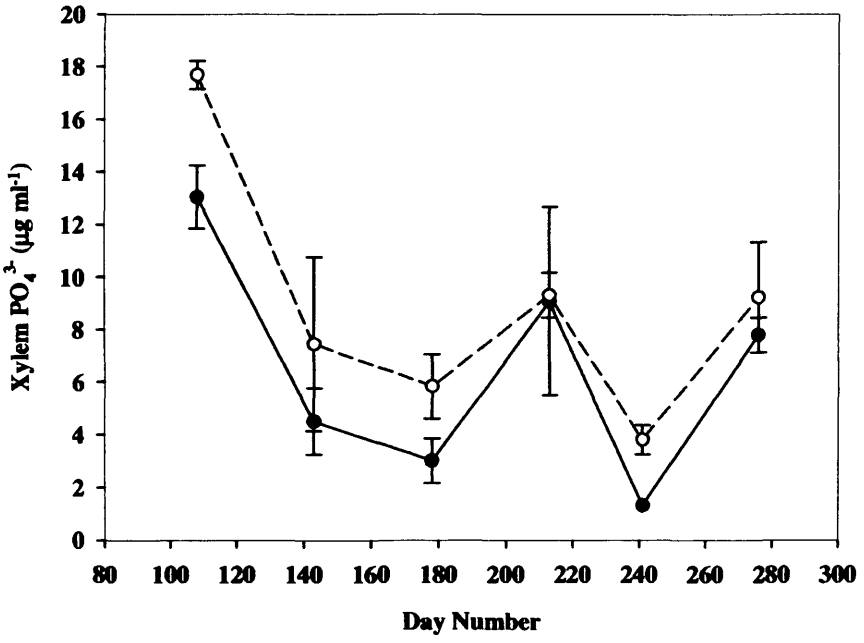


Figure 4.8. Phosphate concentrations in the xylem sap of *S. nigra* trees at the motorway (solid line) and the control site (dashed line) in 2002. Sampling commenced on 18/04/02 and ended on 03/10/02. Data points are the average of 3 replicates SE bars are shown.

Differences in concentrations although not significant appear to be higher at the control site. It has been suggested that PO_4^{3-} might be used as a counter ion to maintain charge balance in the xylem when NO_3^- uptake is restricted, perhaps as the result of N pollution (Chapin *et al.*, 1986; Schulze, 1989). The results in Figure 4.8 would fit well with this hypothesis if it were not for the fact that NO_3^- concentrations in the xylem of *S. nigra* remain unaffected by site differences (Figure 4.2.b). A further explanation is a reduction in soil P availability at the motorway site as the result of N additions (Harrison, 1978, Harrison *et al.*, 1988; Carreira *et al.*, 1997). However, although results in Figure 4.8 may indicate enhanced PO_4^{3-} uptake at the control site, tissue total P concentrations remain unaffected by site differences (Section 3.2.5).

4.2.7. Conclusions

It is important to investigate the effects of N deposition on physiological processes since these may occur prior to the more outwardly visible effects such as injury symptoms and changes in species diversity (Jung *et al.*, 1997). Increased knowledge in this topic may allow physiological changes to be used to monitor the health of vegetation and identify habitats at risk. Results in chapter 3 pointed towards enhanced deposition of N and foliar uptake by roadside trees. The central aim of this chapter was to follow on from this work by investigating how this traffic-derived N impinges on plant metabolic processes. As well as investigating concentrations of N compounds in the leaves and xylem sap of trees we have also measured tissue sugar concentrations and xylem PO_4^{3-} concentrations as these have previously been reported to be affected by N deposition.

S. nigra is a highly nitrophilous pioneer species (Clough, 1993) and as might be expected high amounts of NO_3^- were observed in both the tissue and the xylem of these trees with concentrations peaking during spring. Atmospheric inputs have no effect on xylem NO_3^- although xylem amino acids are higher at the control site than at the motorway site during springtime. It is possible that N transfer from the roots to the shoots is decreased at the motorway site because the N demands of the shoot are being satisfied by atmospheric contributions from the traffic. The motorway however appears to have no effect on the amount of soluble sugars in the leaves. Phosphate concentrations in the xylem do appear to be affected by proximity to the road with increased amounts of this nutrient in the xylem of trees at the control site. This however does not fit with the results in chapter 3 that showed total P to be unaffected by the motorway (Section 3.2.5).

The physiological changes associated with senescence of *S. nigra* occur before the outwardly visible signs of leaf yellowing. Senescence seems to be initiated early in the season by a decline in sugar concentration perhaps as the result of reduced photosynthesis. Sugar levels are then seen to increase, possibly due to the degradation of thylakoid lipid membranes. By the last sampling date, at the beginning of October, sugar levels have dropped significantly whereas concentrations of tissue amino acids have increased 3 fold, most likely as the result of degradation of proteins. In addition to N storage as protein, this nitrophilous pioneer stores a proportion of N as NO_3^-, most likely in the vacuole. Cellular concentrations of NO_3^- were seen to increase throughout the growing season.

C. monogyna conversely, exhibits less nitrophilous behaviour than *S. nigra* since amounts of tissue and xylem NO_3^- are lower. Levels of tissue NO_3^- peak early in the growing season, around mid-April. These high concentrations may occur as the result of (i) inputs from the phloem in the early stages of leaf development (ii) NO_3^- storage in buds (iii) as the result of influx from the xylem prior to the first sampling date. As xylem NO_3^- concentrations are low it is proposed that this species relies mainly on amino acids for transport of N from the roots to the shoots. Xylem amino acid concentrations remain at a constant level in the xylem throughout the year. This, in combination with the low levels of tissue NO_3^-, indicates that this species relies mainly on N assimilation in the roots. Senescence is characterised by a steady increase in tissue amino acid concentrations as well as a late seasonal decline in soluble sugar concentrations. These changes appeared more slowly at the motorway site suggesting that perhaps proximity to the motorway is influencing the onset of senescence.

Despite results in Chapter 3 pointing towards the contribution of traffic-derived N to foliage this Chapter has shown few effects of NO_x deposition on tree physiology. The elevated growth at this site, as demonstrated in Section 3.2.4 may be partly responsible for the lack of differences between the two sites since growth can have a diluting effect, causing metabolite pools to remain at a nearly constant level. However, the fact that few physiological effects of N deposition were observed does not mean that trees are unaffected by traffic emissions throughout the UK since extrapolation from a single field experiment at a specific site to the wider countryside can raise significant problems. For example, the response of trees to N deposition at Mardley heath which is a lowland deciduous

woodland in the S.E. of England may not be applicable to a woodland in the N.W. of England where soils are more likely to be nutrient limited due to the poor nutrient status of the soils.

CHAPTER 5

NITRATE REDUCTASE ACTIVITY AS A BIOMARKER FOR NITROGEN DEPOSITION

5.1. Introduction

5.1.1. Nitrate Reductase biochemistry

The cloning of the NR gene by Campbell and Kinghorn (1990) has led to a wealth of information regarding the structure and regulation of this cytosolic enzyme (for a recent review see Kaiser and Huber, 2001). NR is a homodimeric enzyme made up of two identical NR proteins that function separately in the reduction of NO_3^- (Marschner, 1995). A second more controversial function of NR may be the reduction of NO_2^- to NO (Yamasaki and Sakihama, 2000), but the importance of this activity is relatively small when compared to NO_3^- reduction (approximately 1% of total NR reactions) (Kaiser and Huber, 2001).

The NR protein is approximately 900 amino acids in length and weighs around 100kD. The enzyme has two active sites – the electron donor site and the electron acceptor site. At the donor site NAD(P)H donates electrons to FAD. The electrons are transferred via the heme-Fe to molybdate at the acceptor site. It is at the acceptor site where NO_3^- is reduced to NO_2^-. The enzyme has two-site ping-

pong (oxidized-reduced) steady state kinetics. The basic structure of NR protein is shown in Figure 5.1.

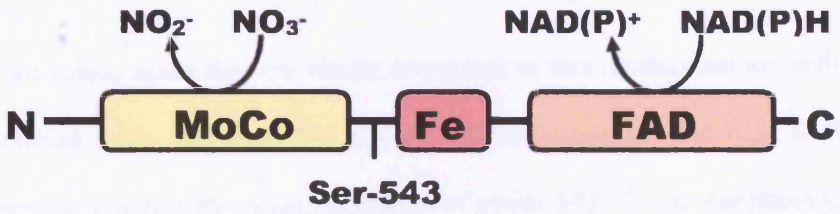


Figure 5.1. Model of the basic NR structure. The N and C terminals are labelled and designated N and C respectively. The enzyme consists of three different functional and structural domains, which are connected by two hinge regions. The serine-543 phosphorylation site is located in hinge-1 and three co-factor regions are shown within the structure. (Modified from Campbell, 1996)

Due to the toxic nature of NO_2^-, the reduction of NO_3^- by NR is under strict regulation. Control is complex and is achieved by manipulation of gene expression, enzyme activity and protein degradation. Gene expression is strongly influenced by NO_3^- availability with transcription occurring soon after application of NO_3^- to the rooting medium (Solomonson and Barber, 1990).

Regulation of catalytic activity involves the phosphorylation and subsequent binding of a 14-3-3 protein. As a result NR is found to exist in 3 states (Kaiser and Huber, 2001):

1. Active free NR
2. Active phosphorylated NR (pNR)
3. Inactive pNR:14-3-3 complex

Two amino acids that are vitally important to this mechanism are serine 543 and arginine 540. Arginine 540 allows ATP to recognise and bind to the enzyme thereby allowing the phosphorylation of serine 543. Once phosphorylated, 14-3-3 proteins may bind with NR to form the inactive complex. It has been proposed that inactivation may occur by a change in NR conformation that interrupts electron transport along the enzyme (Kaiser and Huber, 2001)

The signals for NR degradation are the topic of much ongoing research. It has been established that sugar content plays some role in NR degradation (Cotelle *et al.*, 2000). High sugar concentrations promote expression of the NR gene, block protein kinases and prohibit degradation, the end result being an increase in the amount of active NR (Kaiser and Huber, 2001). It is the inactive form of NR (bound to 14-3-3s), rather than the free active NR that is believed to be susceptible to degradation (Kaiser and Huber, 2001). This idea was supported by an experiment that showed NR protein was more stable when activated (Kaiser *et al.*, 1999). Some confusion still exists regarding the mechanism since Cotelle (1999) showed that in sugar-starved plants not only was degradation increased but binding of the 14-3-3 proteins was inhibited. This seems to imply that it is not the inactive 14-3-3 bound NR that is degraded. To explain this Kaiser and Huber (2001) have proposed that NR degradation may occur by two different proteases. These authors suggest that one protease is active and acts by slowly degrading

inactive pNR:14-3-3 complex whereas another less specific protease is responsible for the breakdown of free NR during sugar starvation.

5.1.2. Effects of the environment on NR activity

N assimilation and NR activity are closely linked to soil NO_3^- availability with NR being induced by the addition of NO_3^- to N-starved plants within a few hours (Oaks *et al.*, 1972). However, the requirement of C skeletons for amino acid synthesis means that N assimilation needs not only to be closely linked to NO_3^- availability but also photosynthesis (Marschner, 1995). Field and Mooney (1986) showed that photosynthesis rates were directly proportional to leaf N content. In order to coordinate NO_3^- reduction with other physiological processes such as photosynthesis plants rely on both environmental and physiological factors to modify NR activity. Environmental factors including light, temperature, drought, time of day as well as internal physiological factors such as hormones, carbohydrates, amino acids, urea, ammonia and leaf water potential have all been reported to act as signals for the modification of NR activity (Haynes and Goh, 1978; Beevers and Hageman, 1983; Oaks, 1991; Collier *et al.*, 2003).

As a result of such signals, NR activity exhibits both diurnal and seasonal rhythms (Clough, 1993; Man *et al.*, 1999). In most plants NR activity increases from dawn, peaks at around midday and then falls as dusk approaches (Bowsher, 1991). Diurnal activity does not always correlate with the amounts of NR mRNA suggesting that post-translational control, as opposed to transcriptional control mechanisms are operating (Hufton *et al.*, 1996). Seasonal changes in NR mean

that maximal enzyme activities might only occur at a certain time of year. For example, depending on the species, NR activities may be highest in the spring coinciding with bud break or late in the season, just prior to leaf senescence (Clough *et al.*, 1989; Pearson *et al.*, 1989; Clough, 1993).

5.1.3. Effects of foliar deposited N on NR activity

Atmospheric NO_2 may enter leaves where it dissolves in the aqueous apoplastic solution to form NO_2^- and NO_3^- which may then be transported into cells (Wellburn, 1990) (Chapter 4, Figure 4.1). Since the supply of NO_3^- is thought to be the most important factor affecting NR activity (Solomonson and Barber, 1990) atmospheric uptake of NO_2 and subsequent conversion to NO_3^- may well stimulate activity of this enzyme. The induction of NR by gaseous NO_2 was first shown by Zeevaart (1974) using peas (*Pisum sativum* L. c.v. Rondo). Enhanced activity was detected within 10 minutes of fumigation demonstrating the quick response of enzyme activity to enhanced substrate availability. Since this work, the induction of NR activity following NO_2 exposure has been shown in a range of species (Wingsle *et al.*, 1987; Theone *et al.*, 1991; Morgan *et al.*, 1992; Hur and Wellburn, 1994; Srivastava *et al.*, 1994). Conversely, Morgan *et al.* (1992) showed that NO fumigation (as opposed to NO_2 fumigation) caused a rapid decrease in NR activities in bryophytes.

With regards to wet deposition there is some debate as to whether or not NO_3^- deposited to leaf surfaces can enter plants without first being washed to the soil and taken up by the roots (Wellburn, 1990). For instance Norby (1989)

demonstrated the induction of NR in *Picea rubens* trees following fumigation but was unable to show the same effect following NO_3^- misting events. Such work suggests that foliar deposited NO_3^- is unable to access cells. Indeed Wolfenden and Wellburn (1986) reported that while sulphate in rain droplets will significantly increase the amount of intracellular sulphate the same effect is not apparent with NO_3^- rain droplets. On the other side of the argument there are several reports of wet deposition entering leaves and stimulating foliar NR activity (Jacobsen *et al.*, 1989, L'Hirondelle *et al.*, 1992, Pearson and Soares 1995). However in misting experiments it is often difficult to guard against increased N supply to the root. It may be the case that wet deposition can be taken up by leaves but the effects are slower than with dry deposition. For example, in a study where both labelled $^{15}NO_3^-$ and $^{15}NO_2$ were supplied to leaves, the distribution of the label within the plants was identical but it took significantly longer for the wet deposited $^{15}NO_3^-$ to be imported into the mesophyll compared to the dry deposited $^{15}NO_2$ (Oghogharie and Pate, 1972).

The induction of NR following foliar N application may be a species dependent phenomenon (Morgan *et al.*, 1992; Pearson and Soares, 1995). It has been demonstrated that NR is more readily induced in nitrophilous pioneer species than it is in climax species (Pearson and Soares, 1995). Nitrophilous species are characterised by a high foliar N content and NR activity (see Chapter 1, Section 1.4.1.).

It is important to note that the stimulation of NR activity by foliar N feeding may only be a transient response. For example, NR activity of *Picea abies* trees were

3 times higher than the untreated controls following a 24 hour exposure to 60 nl l^{-1} NO_2 but when the experiment was allowed to continue for 3 days, enzyme activity declined to the level of the controls. Changes in NO_3^- transport induced by fumigation may be responsible for the transient nature of NO_2 enhanced NR activity (Theone *et al.*, 1991). Again, whether elevated N supply to the leaves is shown to have a transient or a sustained effect on NR may be related to which plant species is tested. Therefore, while stimulation of NR by foliar supplied N has been demonstrated to be a transient phenomenon in the coniferous species, *Picea abies* (Theone *et al.*, 1991), *Picea rubens* and *Pinus sylvestris* (Wingsle *et al.*, 1987) this may not be the case with all species.

5.1.4. Aims and Objectives

Results in Chapter 3 suggested that the assimilation of traffic-derived N was occurring in trees at the motorway site. This Chapter aimed to follow on from this work by looking at the effects of N deposition on foliar NR activities at Mardley Heath. Stimulation of foliar NR activity at the motorway site was used to infer the uptake pathway of the traffic-derived N i.e. whether it was being taken up directly by the leaves or by the roots following deposition. This Chapter thus aims to partly fulfil the second aim as set out in chapter 1 (Section 1.9), to investigate the effects of increased N deposition on foliar uptake and tree physiology. Another aim of this Chapter was to assess the potential of using NR as a biomarker for N deposition. Measurements of NR activity may offer a useful biomonitoring technique for NO_x deposition since this enzyme is common to all

higher plants, substrate inducible, easily assayed and is the rate limiting step in the assimilation of NO_3^- (Guerro *et al.*, 1981).

5.2. Results and discussion

5.2.1. Comparison between foliar NR activities of different species

All species assayed possessed readily detectable leaf NR activities in the field (Figure 5.2). However it is clear that there was much variation between species with respect to NR activity. NR activity in the four woody species analysed was in the following order:

S. nigra > *B. pubescens* > *C. monogyna* > *Quercus spp*

The highest rates of leaf NO_3^- reduction were observed in *S. nigra* and *B. pubescens*. These are pioneer species that are characteristic of the early stages of forest growth. High foliar NR activity observed in this species is in agreement with the findings of many other workers who have reported a preference for NO_3^- uptake and foliar assimilation in pioneers (Smirnoff *et al.*, 1984; Clough *et al.*, 1989; Clough, 1993, Pearson and Soares, 1995).

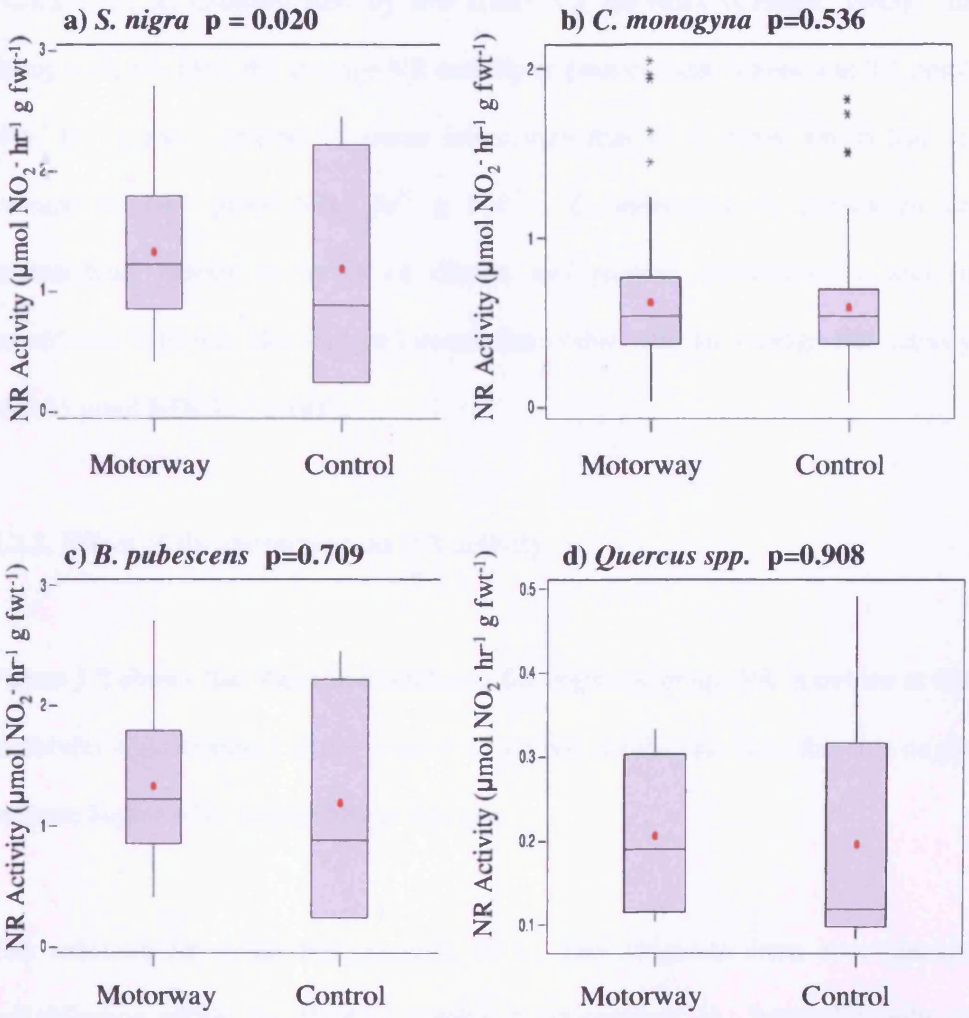


Figure 5.2. Foliar NR activity of (a) *S. nigra* (b) *C. monogyna* (c) *B. pubescens* and (d) *Quercus spp.* Results are the amalgamation of 1 year sampling for *B. pubescens* and *Quercus spp.* and the amalgamation of 3 years sampling for *S. nigra* and *C. monogyna.* The red spots represent the mean values and the horizontal lines within the boxes represent the median values. Outliers are represented by an * . Note the differences in the NR activity scales shown.

It is generally accepted that climax species have a lower capacity for foliar NO_3^- assimilation, as demonstrated by low foliar NR activities (Clough, 1993). In fitting with this idea, the average NR activity of *Quercus spp.* leaves was 0.2 μmol NO_2^- hr^{-1} g fwt^{-1}, around 10 times lower than that of *S. nigra* which had an average of 1.84 μmol NO_2^- hr^{-1} g fwt^{-1}. *C. monogyna* is considered an intermediate species in terms of climax and pioneer characteristics and in accordance with this idea had an intermediate value with an average NR activity of 0.63 μmol NO_2^- hr^{-1} g fwt^{-1}.

5.2.2. Effect of the motorway on NR activity

Figure 5.2 shows that there is a tendency for higher average NR activities at the motorway site compared to the control. Since NR is substrate inducible this might indicate higher NO_3^- availability at this site.

The substrate for foliar NR enzyme, NO_3^-, may originate from root uptake, redistribution within the plant or uptake of atmospheric N. Internal supply of NO_3^- to the leaves was investigated by assaying the xylem sap of the trees (section 4.2.1). There was no significant difference between the sites with respect to NO_3^- concentrations in xylem sap. This indicates that differences in root-shoot transfer are not responsible for the observed differences in foliar NR activity however it is important to note the rate of xylem flux (transpiration) was not measured and therefore our measurements of xylem sap NO_3^- may not accurately represent NO_3^- supply to the shoots.

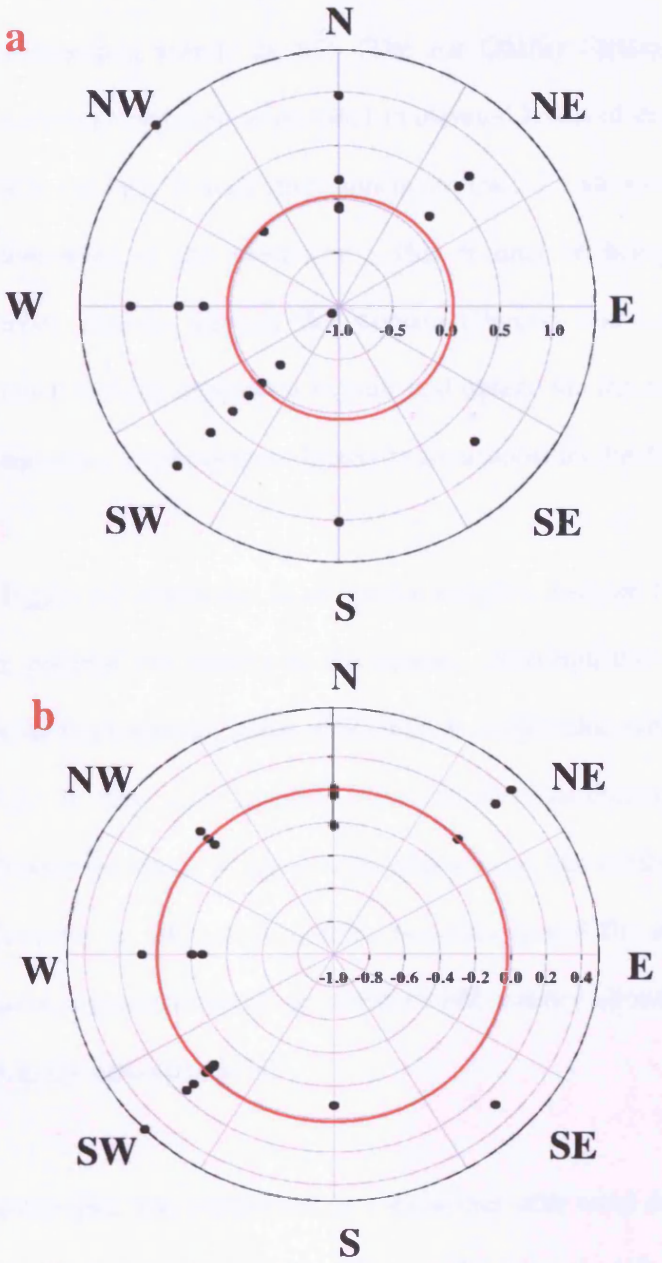


Figure 5.3. The effect of wind direction on NR activity of (a) *S. nigra* and (b) *C. monogyna* at Mardley Heath during 2001 and 2002. Data points represent the difference between the NR activity at the motorway and the control sites. The radial axis is the difference between NR activity at the polluted and control sites (μmol NO_2^- hr^{-1} g fwt^{-1}).

NO_x produced by cars travelling on the road will be quickly deposited in the downwind environment mainly as NO_2 (The Air Quality Strategy). Therefore, trees at our motorway site may be exposed to elevated levels of dry deposition in the form of NO_2 and NO. Indeed the results in section 3.2.1 showed elevated NO_2 deposition downwind of the motorway. This N may be being taken up by vegetation either directly through the stomata (Hanson and Lindberg, 1991) (section 4.1) or following deposition to soils and uptake via the roots (Wellburn, 1990). In either case, traffic derived N may become substrate for NR enzyme.

The data in Figure 5.2 shows that in all species sampled, average NR activity was higher at the polluted site relative to the control. Although this difference was insignificant for most species, when considered in conjunction with the $\delta^{15}N$ data (section 3.2.2.) it may point towards plants at the contribution of motorway derived N to plant nutrition. *S. nigra* is the only species that exhibits a significant difference between the NR activities at the two sites ($p=0.020$). As a pioneer, *S. nigra* may have a greater capacity to stimulate NR activity allowing the plant to utilise or 'mop-up' atmospheric N.

The NR activity data was considered in conjunction with wind direction (Figure 5.3). Wind direction may affect the difference between the NR activities at the two sites either by altering the supply of atmospheric NO_x from the traffic to the trees. Figure 5.3 shows the difference between NR activities of *S. nigra* and *C. monogyna* at the motorway and the control sites. Where values are positive (i.e. data points are outside of the red circle) this represents higher activities at the motorway site relative to the control. In Figure 5.3.a most of the points are

outside of the red circle, showing a tendency for foliar NR activities to be higher in *S. nigra* individuals located next to the motorway than at the control site. Results for *C. monogyna* in Figure 5.3.b show roughly equal numbers of points inside and outside of the circle again demonstrating the fact that there is little differences in NR activity at the two sites for this species. While Figure 5.3 does demonstrate the prevalence of a south westerly wind at this site it does not provide much information regarding the impact of wind-direction on NR activity since unequal sample numbers make comparisons difficult.

Although wind strength was not accounted for in this analysis this too may have had some bearing on the difference in NR activities between the sites, perhaps by affecting the supply of gaseous N to the leaves. In high wind speeds, traffic derived atmospheric N may have been transported further distances, leading to greater NR activity at the control site and consequently lower differences between the sites. On calm days on the other hand, trees at the control site will receive less traffic derived NO_x although trees at the motorway site will still receive this gaseous N due to turbulent air flows caused by the passing traffic.

5.2.3. Seasonal trends in NR activity

It is important to note that the results presented in Figure 5.2. are the amalgamation of measurements of NR activity made over several years. While this has the advantage of smoothing variation, emphasising general mean values as well as stressing the range of activities it fails to account for seasonal variations in NR activity which can be large and significant (Clough, 1993).

The seasonal profiles of NR activity in *S. nigra* leaves activity during 2001 and 2002 are shown in Figure 5.4. NR activity was observed to increase rapidly following bud-opening reaching a peak in late May (day 143). Activity then decreases during the growing season before rising again in autumn. The decline in NR activity throughout the growing season is likely to be due to changes in foliar NO_3^- concentrations within the plant (Section 4.2.2.) since the level of enzyme activity is a function of NO_3^- concentration (Solomon and Barber, 1990).

The NR activity peak during early May corresponds to a period when *S. nigra* was one of the few woody plants in leaf. NO_3^- reduction is closely linked to photosynthetic activity and supply of fixed C (Section 5.1.2), so this early spring flush could be due to high light availability in the absence of the tall tree leaf canopy. High NR activity around day 140 could also be related to increased N availability in the soil. In temperate zones there is often a peak in microbial activity around late winter or early spring (Fitter and Hay, 1991). This leads to flushes of soil NO_3^- and NH_4^+ which may stimulate NR activity during the spring.

Other factors that may be involved with the spring flush of NR activity are leaf age and remobilisation of stored N. It is generally accepted that NR activity is highest in young, actively growing tissues such as those sampled during the springtime (Kenis *et al.*, 1992; Gonzales-Real and Bailler, 2000).

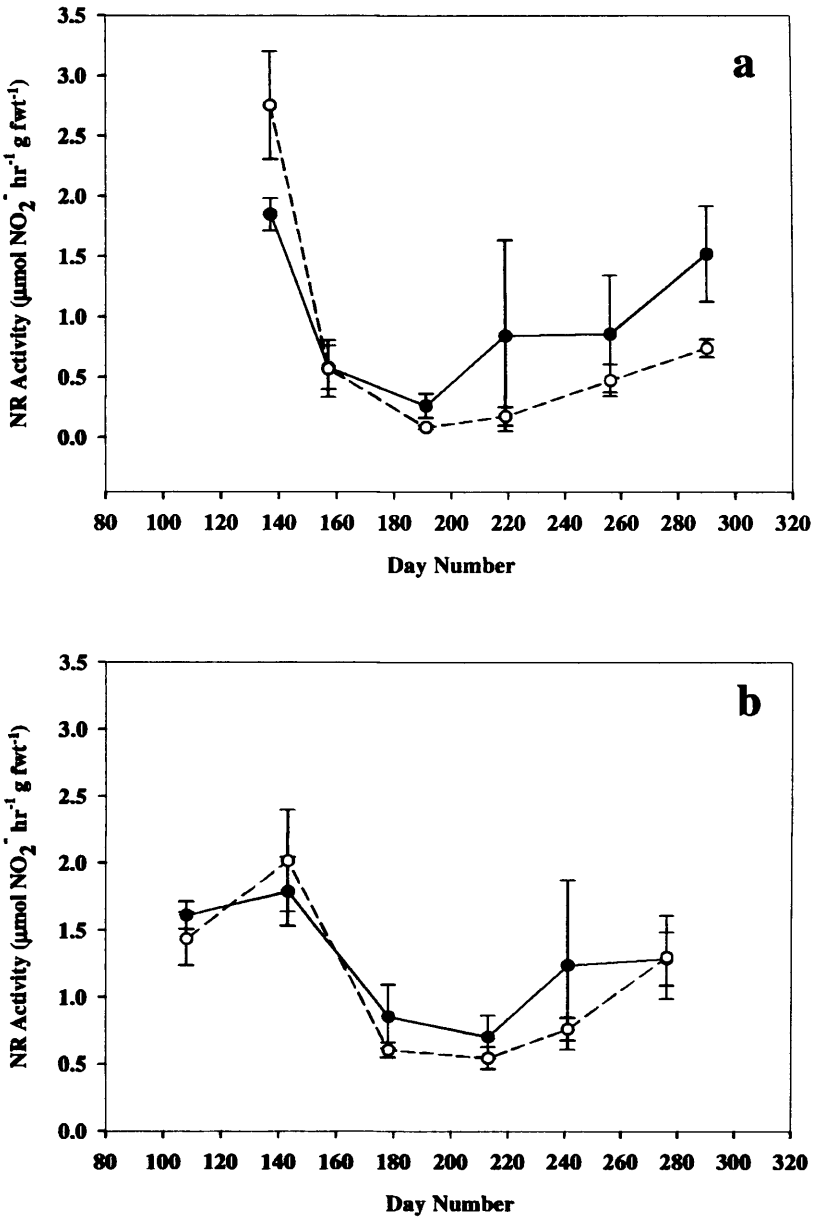


Figure 5.4. Seasonal profiles of NR activity in *S. nigra* foliage during (a) 2001 and (b) 2002 at the motorway (solid line) and the control site (dashed line). Data points are the average of 3 replicates. SE bars are shown. Sampling commenced on 17.05.01 and 18.04.02.

In an effort to conserve N, many plants store N in woody tissues before leaf fall. This N is remobilised in the spring and can account for up to 40% of the total plant N (Stephens *et al.*, 2001). Therefore, the observed spring peak in NR activities in the leaves of *S. nigra* may be caused by a combination of factors, that is to say, high light availability, an increase in soil NO_3^-, the induction of NR in young tissues and the remobilisation of stored N. Diurnal changes are not a factor in these results because sampling was always conducted at the same time of day.

C. monogyna also exhibited marked seasonal variations in terms of leaf NR activity (Figure 5.5.). The seasonal profile was quite different from that observed for *S. nigra* (Figure 5.4.). In *C. monogyna* relatively low levels of NR activity were observed in leaves until near the onset of autumn.

Pearson *et al.*, (1989) outline three possible causes of late seasonal increases in NR activity namely an increase in flux of NO_3^- from the root to the shoot, the release of vacuolar stored NO_3^- and the removal of an end product inhibitor. The first hypothesis does not seem likely as neither tissue nor xylem NO_3^- concentrations showed no late seasonal increases (Section 4.2.2). Although it should be remembered that actual fluxes to the leaves were not measured. It is not possible to comment on the second hypothesis since this work did not encompass separate measurements of vacuolar and cytoplasmic NO_3^-. A further possible explanation for late seasonal increases in NR activity may reflect another function of the enzyme, that of NO generation (Yamasakia and Sakihama, 2000) since increases in NO may be involved in triggering senescence (Pedroso *et al.*, 2000).

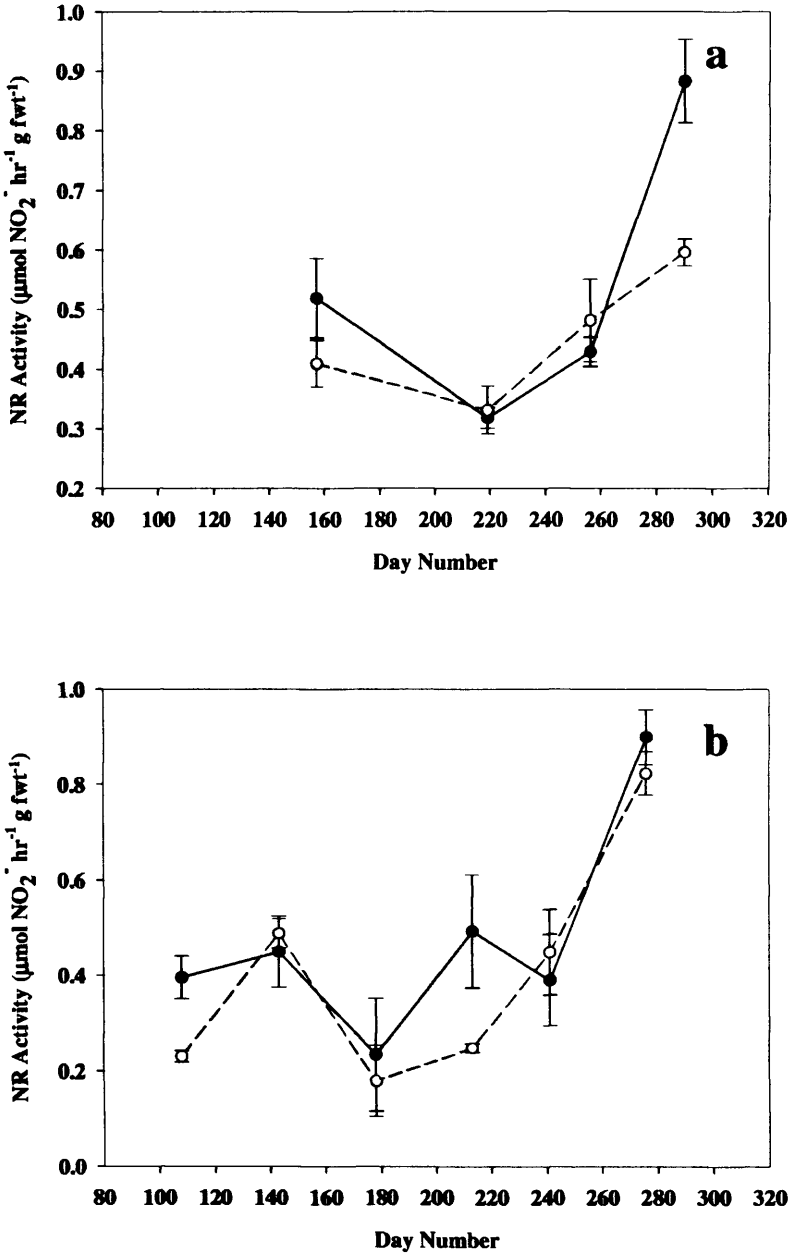


Figure 5.5. Seasonal profiles of NR activity in *C. monogyna* foliage during (a) 2001 and (b) 2002 at the motorway (solid line) and the control site (dashed line). Data points are the average of 3 replicates. SE bars are shown. Sampling commenced on 05.06.01 and 18.04.02.

5.2.4. *In vivo* NR assay method - plus or minus KNO_3

In most trials where NR activity has been assayed *in vivo*, the assay is performed using a medium containing an excess of KNO_3 ($+KNO_3$ assay). NR activity is then measured by examining the amount of NO_2^- that is produced during incubation. However, according to certain authors, *in situ* NR activity is most closely approximated by an assay without exogenous NO_3^- ($-KNO_3$ assay) (Andrews, 1986; Thomas and Hilker, 2000). In order to investigate this whether this $-KNO_3$ method has added potential over the $+KNO_3$ method *S. nigra* leaves collected from the both motorway and the control sites were examined for NR activity using the two types of assay i.e. one using an assay medium containing KNO_3 substrate ($+KNO_3$) and one that did not ($-KNO_3$). This comparison measures to what extent the tissue nitrate pools are limiting the NR activity and may provide a more appropriate bioassay for atmospheric N deposition by providing a direct link between these two physiological parameters.

Five individual trees of each species were sampled at each site. One leaf from each tree was used to provide material for one of each assay mediums in order to reduce variation due to tree and leaf differences. For the $-KNO_3$ assay the rate of production of NO_2^- is dependent on two factors, namely the activity of the enzyme and the amount of NO_3^- substrate within the tissue. The $+KNO_3$ assay however will only be dependent on the activity of the enzyme since the assay medium is saturated with KNO_3 and all other substrates for the reaction are supplied in excess. The results of these two types of NR assay are shown in Figure 5.6.

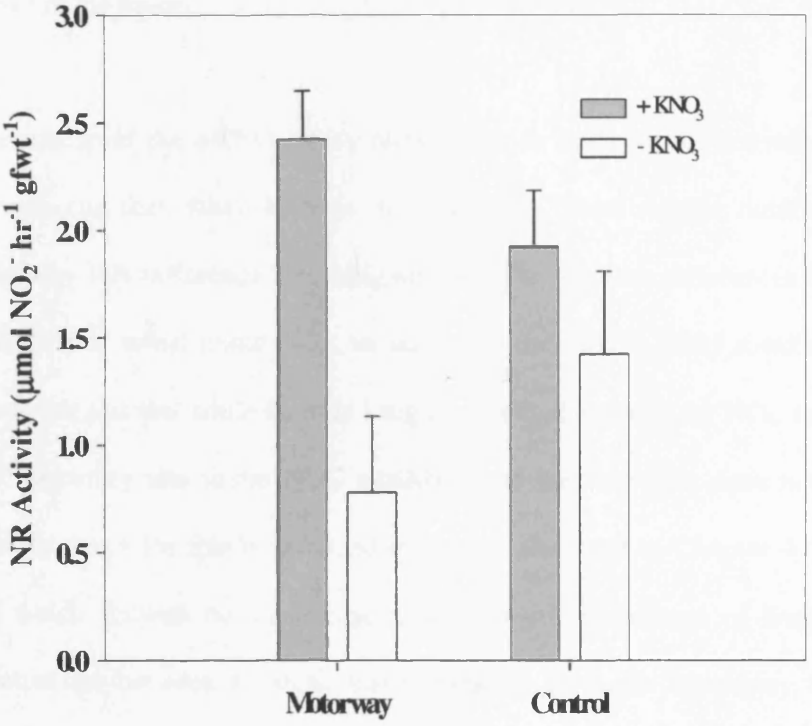


Figure 5.6. *S. nigra* foliar NR activity. Assay carried out with and without additions of KNO_3 to the assay medium. Data points are averages of 5 replicates. SE bars are shown. Sampling took place mid- July.

Differences between the $+KNO_3$ and $-KNO_3$ are likely to be due to lower amounts of NO_3^- in the $-KNO_3$ assays and therefore the production of NO_2^- by NR is less than when the assay is performed with the addition of KNO_3 substrate. The $-KNO_3$ assay results at both sites exhibit much more variation than the $+KNO_3$ assays. Enhanced variation is probably the result of the extra variable in

the experimental conditions introduced in the $-KNO_3$ assay, namely the amount of substrate in the tissue.

The results from the $+KNO_3$ assay alone seem to show higher activities at the motorway site than when NO_3^- is not limiting. Small sample numbers may explain why this difference was insignificant. The were no differences between assimilation of actual tissue NO_3^-, as shown by the $-KNO_3$ assay results. This seems to suggest that while there is a higher potential capacity for NO_3^- reduction at the motorway site tissue NO_3^- availability at the two sites does not differ. Further evidence for this is provided by results presented in Chapter 4 (Section 4.2.2) which showed no significant differences in the amount of tissue NO_3^- concentrations between *S. nigra* leaves sampled from the motorway and the control sites over several months.

Why then would the capacity for NR assimilation be highest in *S. nigra* leaves at the motorway site when there appears to be no elevated tissue NO_3^- concentrations? One possible explanation is that by removing the leaves from these roadside trees one is effectively removing one of their sources of NO_3^-. Another contributory factor may be that during the time between leaf abscission and the start of the NR incubation (in some cases up to 2 hours). High NR activity in leaves collected at the motorway site may have caused the amount of NO_3^- substrate in the tissues to decline before the $-KNO_3$ NR activity was assayed.

The results presented in Figure 5.6 indicates that the exclusion of KNO_3 from the assay medium is not a useful technique for increasing the accuracy of the NR assay since the $-KNO_3$ assay did not show a difference between the motorway and the control site and excluding KNO_3 from the assay medium appears to increases the variability of results.

In future work it may be useful to further investigate the activation state of NR. This may be done by carrying out the in vitro assay with and without Mg^{2+} additions. However, despite numerous attempts, a stable extract was not obtained and the results were deemed too unreliable to present as part of this thesis.

5.2.5. Estimation of the contribution of traffic derived N to plant nutrition

NR is an important step in the assimilation of atmospheric NO_x. Therefore by subtracting the foliar NR rate of control plants from that of plants exposed to a pollution source a rough estimate of the contribution of atmospheric N to plant nutrition may be obtained. This method was applied to the data collected for *S. nigra* and *C. monogyna* during 2001 and 2002 (Table 5.1). Results of seasonal NR activities in $\mu mol\ NO_2^-\ hr^{-1}\ g\ fwt^{-1}$ were multiplied for each species at each site to give a measurement of the amount of N assimilated over the entire season per ha of leaf material. By subtracting values of individuals at the control site from those at the polluted site it is hoped that we have obtained an estimate of the contribution of atmospheric N to foliar assimilation in these species.

Table 5.1. Estimation of the amount of additional N that is assimilated by foliar NR in S. nigra and C. monogyna during 2001 and 2002.

Species	Year	Amount of additional N assimilated by NR in foliage at motorway site (Kg N ha^{-1} $season^{-1}$)
S. nigra	2001	17.13
	2002	8.12
C. monogyna	2001	2.21
	2002	3.24

The calculation was based on the conservative assumptions of zero activity at night and a twelve hour day. Table 5.1 shows that the small differences in NR activity may be contributing to large amounts of additional N being assimilated by tree foliage at the motorway site, presumably as the result of uptake and assimilation of traffic derived NO_x from the nearby road. This is particularly true for *S. nigra* which, according to the results in Table 5.1 assimilates 17.13 (2001) and 8.12 (2002) Kg N ha^{-1} $season^{-1}$ more at the motorway site than is assimilated at the control site.

The variation between these values may be the due to large fluctuations in enzyme activity according to environmental factors. Large fluctuations are especially apparent in nitrophilous species such as *S. nigra* that exhibit high maximal NR activities. The amount of additional NO_3^- assimilation estimated to be taking place in the leaves of *C. monogyna* at the motorway is quite low implying that this

species has a low capacity for foliar uptake and assimilation of traffic derived NO_x. *S. nigra* plants may be useful N sinks in heavily NO_x polluted environments.

5.2.6. Intensive NR sampling

Intensive field sampling using 5 replicates of *S. nigra* and *C. monogyna* was performed during early 2004. The results are presented in Figure 5.7. The early seasonal peak for *S. nigra* NR activities is once again apparent (Figure 5.7.a). At this time of year differences between foliar NR at the two sites are small indicating that this would not be a useful time of year to use *S. nigra* NR activities as a biomarker for N deposition. Conversely, *S. nigra* foliar NR activities may be useful biomarkers from the end of May onwards foliar rates at the motorway and the control site appear to diverge after 26/05/04.

Results for *C. monogyna* (Figure 5.7.b) confirm what has already been demonstrated for this species i.e. low foliar NR activity and few differences in activities at the motorway site and the control site. *C. monogyna* therefore appears to offer little potential in terms of using foliar NR activities as a biomarker for N deposition.

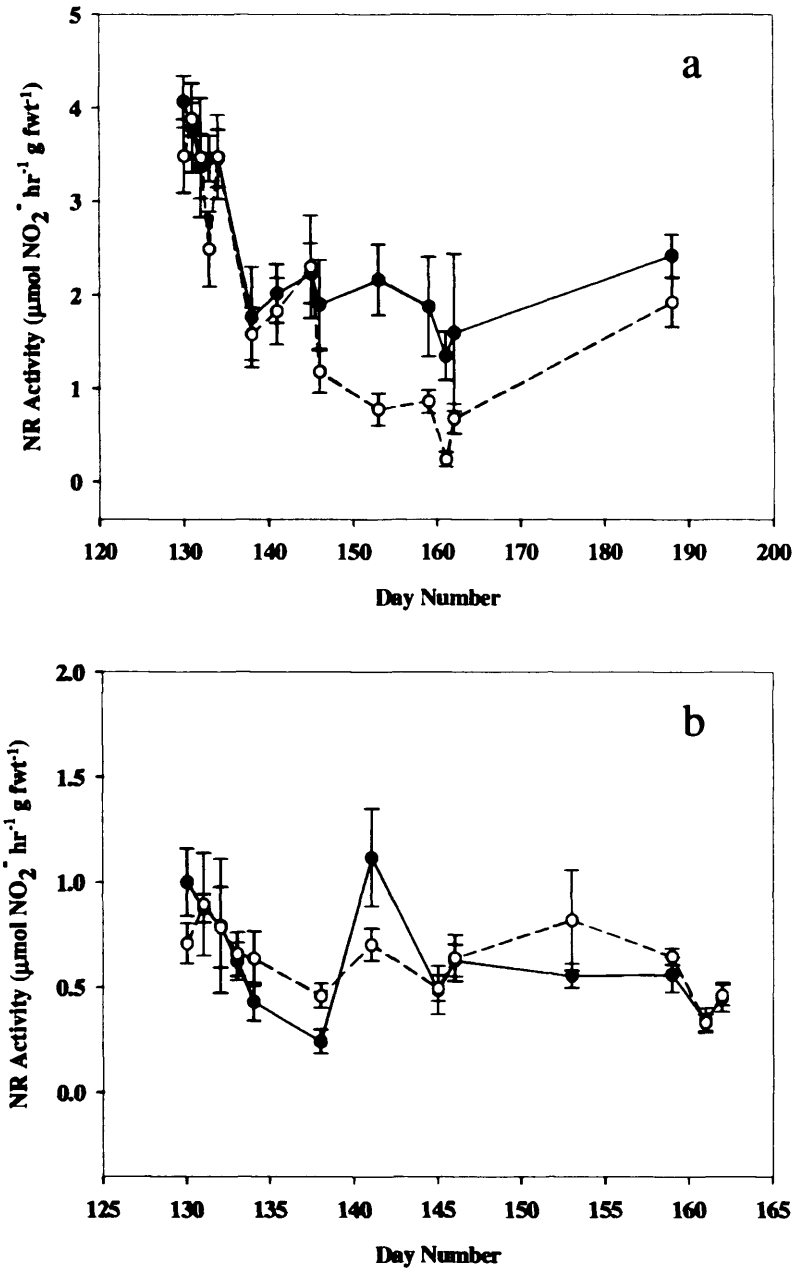


Figure 5.7. Profiles of NR activity during 2004 in (a) *S. nigra* foliage and (b) *C. monogyna* at the motorway (solid line) and the control site (dashed line). Data points are the average of 5 replicates. SE bars are shown. Sampling commenced on 10.05.04.

5.2.7. Conclusions

As discussed previously, tree species differ in terms of NR activity and activity measurements can be used to classify trees as either nitrophilous pioneers or climax species (Section 1.4.1). The NR activities reported in this Chapter support previous classifications of the species examined as either pioneer species (*S. nigra* and *B. pendula*), climax species (*Quercus spp.*) or intermediate species (and *C. monogyna*) (Clough *et al.*, 1989; Clough, 1993; Pearson and Ji, 1994). The ability to induce NR with in response to N deposition appears to be associated with high NR activities as *S. nigra* not only exhibited the highest foliar NR activities, but was the only species to show a significant difference between activities at the motorway and the control sites ($p=0.020$). An elevated activity in *S. nigra* at the motorway site compared with the control is likely to be due to the additional assimilation of traffic-derived N which may be entering the leaves via the stomata.

It may be concluded that NR activities of *Quercus spp.*, *C. monogyna* and *B. pendula* do not respond significantly to elevated N deposition at Mardley Heath. It therefore seems unlikely that these species are taking up and assimilating substantial amounts of traffic-derived NO_x at the motorway site via foliar uptake. Results from previous Chapters however have pointed towards the contribution of traffic-derived NO_x to plant nutrition and growth of trees. It must therefore be assumed that traffic-derived NO_x is entering the plant following deposition to the soil and uptake by the roots.

S. nigra on the other hand does show elevated NR activities in leaves at the motorway site compared to the control. High foliar NR activities may allow *S. nigra* leaves to act as NO_x sinks, removing NO_x from the atmosphere by foliar uptake. *S. nigra* may have potential for cleansing the atmosphere around point sources of NO_x and also for foliar NR activities to be used as biomarkers of N deposition.

The idea of using NR as a biomarker is not new since both Kryult *et al.* (1994, 1996) and Norby (1989) have previously looked at this topic. These workers found although NR activity responded rapidly to changes in atmospheric N when fumigated under laboratory conditions it could not be used as a reliable biomarker in the field. One of the major problems these authors found was the large number of factors other than NO_3^- availability that can influence NR activity in the field. Indeed, the results presented in Figures 5.4, 5.5 and 5.7 show much seasonal variation in terms of NR activity.

The results presented in this Chapter indicate that further work should be conducted into the use of NR as a biomarker, taking into account time of year for sampling, which species is used and other measures that may be taken in order to reduce variation. For instance this study aimed to reduce some of the variation caused by diurnal changes by always sampling at the same time of day. This Chapter has shown sampling month to be important since Figures 5.4 and 5.7.a seem to suggest the response of *S. nigra* NR activity to NO_x is largest during the summer when the motorway and control profiles diverge. In the study by Krult *et al.* (1996) NR activity of *Pinus ponderosa* needles were sampled only once during

the summer. This may have influenced their results as this species may not respond at this time of year for example due to water shortages modifying stomatal behaviour. Species choice is also very important to consider. Out of the four trees examined in this Chapter, only one of these, *S. nigra* appeared to offer potential as a useful NR biomonitoring species. Coniferous species were used by Kryult *et al.* (1994, 1996) and Norby (1989) who looked NR activities in *Picea rubens* and *Pinus Ponderosa* however coniferous species tend to have low capacities for NO_3^- reduction in the shoots (Peason *et al.*, 1989; Smirnoff *et al.*, 1984; Pearson and Soares, 1995) and where N has been found to stimulate NR activity this response is often only a transient phenomenon (Wingsle *et al.*, 1987; Theone *et al.*, 1991). It is recommended that in order to establish NR as a reliable biomarker further work is focused on activities in *S. nigra* during the summer time.

CHAPTER 6

DEPOSITION OF ATMOSPHERIC PARTICLES TO LEAF SURFACES

6.1. Introduction

6.1.1. Deposition of particles to vegetation

Airborne particulates may be deposited to vegetation. Deposition to vegetated surfaces is influenced by not only by prevailing weather conditions and topography but also by surface characteristics. Rough surfaces are believed to capture more deposition than smooth surfaces (Farmer, 2002). Surface wetness is also a factor with wet surfaces receiving more deposition than dry surfaces (Farmer, 2002).

Particle size is important in determining deposition velocity. Fine particles are deposited slowly and hence can be transported over long distances (Allen *et al.*, 2001). Particle size is also important in determining whether particles remain on the plant surface or enter the leaf. Since open stomata generally have a diameter of between 8 – 10 μm only particles smaller than this are able to enter inside leaves. Particles of a certain size range may block stomata thereby preventing transpiration (Singh and Rao, 1981). However, since stomata tend to be prevalent on the lower surfaces, these effects require turbulent motion around and below the

leaf (Farmer, 2002). Other detrimental effects of particulates on plants include shading that may lead to increased leaf temperatures, reduced photosynthesis and an increase in chlorophyll (Eller, 1977).

6.1.2. Surface nitrate

Dry NO_x deposition may be deposited onto the foliar canopy, leading to accumulation on the foliage (Bytnerowicz *et al.*, 1991) which in turn may increase the N input to the forest floor by stemflow. Stemflow is the spatially localized point input of precipitation and solutes at the base of the plant stem that occurs as rainwater is channelled via branches and the stem to the soil. Surface deposition of NO_3^- may also play an important role in tree damage, as when dissolved in surface moisture it can be severely acidic and can cause tissue injury with leaching of cations such as K^+, Ca^{2+} and Mg^{2+} from the foliage (Scherbatskoy and Tyree, 1990). Dry deposits of NO_x are thus considered to be a contributory factor to forest damage (Chiwa *et al.*, 2003).

Several workers have attempted to estimate N deposition by measuring the NO_3^- amounts accumulated on leaf surfaces (Hanson and Lindberg, 1991; Bytnerowicz *et al.*, 1991; Sanz *et al.*, 2002; Chiwa *et al.*, 2003). Foliar rinsing techniques are often used in conjunction with the surface area of the rinsed material in order to obtain an estimate of the rate of dry deposition. The flux of dry deposition to the forest floor may then be calculated by taking into account the leaf area index (L.A.I) of the trees.

6.1.3. Zinc as a biomarker for traffic flow

The introduction of unleaded petrol has led to a yearly 20% reduction in the Pb PM_{10} concentrations (Monaci *et al.*, 2000). Whereas in the past Pb was used as a marker for exhaust emissions, the phasing out of leaded petrol means that nowadays there is no unequivocal marker for exhaust emissions. In response to this, other elements including Ba, Cu and Zn have received attention as future markers for traffic pollution. Several studies have focused on the use of Zn as a possible biomarker for pollution (Fatoki, 1996; Pearson *et al.*, 2000; Lau and Luk, 2001; Hose *et al.*, 2002)

Zn is used as an additive in lubricating oils as zinc diethyldithiophosphate and as filler in car tyres and brake linings (Harrison, 2003). This Zn may be incorporated into road dusts through oil leakage and combustion, and by mechanical wear of the brakes and tyres. For instance, Harrison (2003) demonstrated that roadside levels of Zn as well as the elements Cu, Mo, Ba and Pb showed significant correlations with NO_x and particle count indicative of a traffic source contribution. The correlation coefficient between Zn and particle number count in this study was relatively high ($r=0.472$) suggesting a high but not exclusive contribution from traffic.

Further analysis of the size distribution of Zn particles close to a traffic source revealed that Zn is evenly distributed through the size fractions (Harrison, 2003). This suggests that multiple sources are contributing to different particle size ranges. Allen *et al.* (2001) came to a similar conclusion (2001) after discovering

Zn present in particle size fractions extending from fine to coarse at several rural and semi rural locations within the UK. Non-traffic sources of Zn particulates include biomass combustion (Nriagu, 1989), combustion of fossil fuels, metallurgical processes (Pacyna, 1986) and the resuspension of surface dusts (Allen *et al.*, 2001).

Scientists have been aware of the correlation between traffic flow and plant Zn content for some time. Elevated Zn in roadside plants was observed in 1970 (Lagerweff and Speltch, 1970). Since then the heavy metal content of plants has been used to estimate pollution on a national (Dmuchowski *et al*, 1995; Berthelsen, 1995), individual city (Monaci *et al.*, 2000; Pearson *et al.*, 2000; Lau and Luk, 2001), and roadside scale (Ylaranta, 1995; Fatoki, 1996). These studies tend to involve the complete digestion of plant material followed by heavy metal analysis of the product.

The benefit of using plants as biomonitors over other monitoring methods is that plants can accumulate heavy metals to concentrations that are much higher than the air. In a study carried out in and around London, average concentrations as high as 805 μg g dwt^{-1} were recorded in the moss, *Bryum argentums* (Pearson *et al.*, 2000). This is significantly higher than the 30 μg g dwt^{-1} Zn commonly present in plant tissues in pollution-free areas. Where studies have examined the relationship between road traffic and several different heavy metals, the highest correlation was found for Zn when compared with Cu (Fatoki, 1996) and Pb (Pearson *et al.*, 2000). Additional care needs to be taken if using Zn as a

biomarker near the sea due to the exchange of Zn with sea-salt cations (Berg *et al.*, 1995).

6.1.4. Atmospheric Zn concentrations in the UK

Zn is an element typically associated with long-range transport (Berg *et al.*, 1995) and consequently may exert effects in regions remote from its source. Furthermore, Zn metal concentrations are higher in air masses of European origin than in those which had only crossed the UK (Chester *et al.*, 1993; Otten *et al.*, 1994; Allen *et al.*, 2001). Recorded atmospheric concentrations of particulate Zn are shown in table 6.1.

Table 6.1. Atmospheric Zn concentrations at UK sites

Author(s)	*Location*	*Site Details*	*Zn concentration (ng m^{-3})*
Baker, 1997	Chilton	Rural	0.36 - 6.0
Allen *et al.*, 2001	Castlemorton	Rural	11
	Brownfields	Semi-rural	63
	Auchencorth	Rural	5.2
Harrison *et al.*, 2003	Birmingham	Roadside	29.9

6.1.5. Zinc Toxicity

As is the case with many heavy metals, root uptake of Zn can have phytotoxic effects on plants. Athar and Ahmad (2002) examined the toxicity of several heavy metals and found the phytotoxic effects were in the following order Cd>Cu>Ni>Zn>Pb>Cr. Metal contaminated soils can result in dramatic decreases in shoot N content (up to 79.4%) as well as a reduction in growth and reproduction (Athar and Ahmad, 2002). Naturally occurring metal ores can cause high local soil concentrations (Fitter and Hay, 1991) as can the contamination from industrial waste or the atmosphere. Release of ions to the soil solution is facilitated by low pH. Zn has enhanced mobility, biological availability and therefore toxicity at pHs < 6.2 (Martinez *et al.*, 1999). N source is also important with plants growing on NH_4^+ suffering from less toxic effects of Zn than NO_3^- grown plants (Smirnoff and Stewart, 1987).

The phytotoxic nature of Zn is cause by its interactions with various chemical groups (Fitter and Hay, 1991). Zn has been shown to cause an inhibition in the activity of certain enzymes. Enzymes will only operate within well-defined ionic limits and any change can cause a decrease in activity. In addition, Zn has specific reactions and is a co-factor required by some particular enzymes, but usually at low concentrations. The toxic nature of Zn may be due to the ability of Zn to form complexes with sulphydryl (SH) and other chemical groups. Brune *et al.* (1995) showed that Zn decreased the amount of SH groups in plasma membrane proteins of barley root cells. Photosynthetic electron transport is also affected by Zn, resulting in the inhibition of CO_2 fixation (DeFilippis, 1981, Van

Assche and Clijsters, 1986). Cellular damage by superoxide radicals may also occur where Zn^{2+} causes a reduction in the cellular ATP and NADPH pool (Luna *et al.*, 2000).

Despite of its toxic effects some plant species appear resistant to high levels of soil Zn. Resistance cannot occur by simple exclusion from cells because Zn is required as an essential micronutrient. Instead resistance seems to occur by amelioration i.e. the plant takes up the ion but acts to minimize its effects. Phytotoxic effects are avoided by certain species by the Zn localization. Several workers have demonstrated that Zn can be accumulated in the root cell walls of resistant species (Peterson, 1969; Turner and Marshall, 1972). In other cases Zn appears localised in the vacuole where it may be further inactivated by chelation with malate (Mathys, 1977). Zn tolerant *Deschampsia cespitosa* accumulates citrate and malate in the roots in response to exposure to Zn (Smirnoff and Stewart, 1987). This process may serve to inactivate Zn by chelating it into a harmless form. Work investigating resistance to another heavy metal, revealed that 97% of Cadmium taken up by *Rauvolfia serpentina* cells was present as phytochelatin complex (Kneer, 1992). Plants that have an active chelating system can actually have an increased Zn requirement (Mathys, 1975). Zn resistance appears to be a multiple phenomenon partly involving exclusion in root cell walls and partly localization in vacuoles (Fitter and Hay, 1991).

Extreme resistance where Zn is accumulated in tissues to concentrations above 10 mg Zn g dwt^{-1} is regarded as Zn hyperaccumulation (Brooks, 1998). Macnair (1999) showed that Zn tolerance and hyperaccumulation are genetically

independent characteristics. *Thlaspi caerulescens* and *Arabidopsis halleri* are well known Zn accumulators, with extraordinarily high Zn concentrations of >1 M found in the shoots of *A. halleri* (Kupper *et al.*, 2000). In this species the Zn appeared to be concentrated at the base of trichomes. This agrees with work carried out on Ni, where an investigation in *Berkheya coddi* revealed Ni accumulation was in the cuticle of the upper epidermis (Zhao *et al.*, 2000).

6.1.7. Aims and objectives

This Chapter aims to fulfil the third aim of this thesis as set out in Section 1.9 i.e. to investigate the deposition of Zn particles and NO_3^- to leaf surfaces as possible techniques for monitoring traffic exposure. Whereas several previous studies have shown total plant Zn to correlate with traffic exposure plant surface Zn has rarely been examined. Indeed in one study, tree leaves were washed prior to analysis thus removing any particulate Zn that may have been present on the leaf surfaces (Monaci *et al.*, 2002). This chapter aimed to investigate the deposition of Zn particles using both total leaf Zn and measurements of surface Zn alone. The suitability of these two techniques for assessing traffic exposure was compared. Contrary to Zn, amounts of surface NO_3^- have been studied by several workers who showed that measurements of NO_3^- may be a useful tool for assessing flux of dry NO_x deposition to woodlands (Bytnerowicz *et al.*, 1991; Sanz *et al.*, 2002; Chiwa *et al.*, 2003). Therefore in addition to looking at Zn, surface NO_3^- was be measured on tree leaves at Mardley Heath. A range of species growing on Marylebone Road, central London were also examined for surface amounts of Zn in order to look at possible differences in particle absorption and retention

between species. Species differences were investigated further using electron microscope techniques.

6.2. Results and Discussion

6.2.1. Surface Nitrate

Surface NO_3^- amounts were measured on tree leaves at the motorway and control sites at Mardley Heath using the foliar rinsing technique described in Chapter 2 (section 2.16). Figure 6.1. shows the results for *S. nigra* and *C. monogyna* leaves over the growing season of 2002. Surface NO_3^- was detected on the leaves of both species ranging from 1.3 – 3.7 and 2.3 – 9.8 μg NO_3^- m^{-2} for *S. nigra* and *C. monogyna* respectively. These values are comparable to the amounts of NO_3^- (ranging between approximately 1- 7 μg NO_3^- m^{-2}) reported by Chiwa *et al.* (2003) on pine trees at Mount Gorkurakuji, western Japan. However, whereas these authors have observed different amounts of surface NO_3^- at polluted and control sites, such differences are not apparent here between the motorway and the control site.

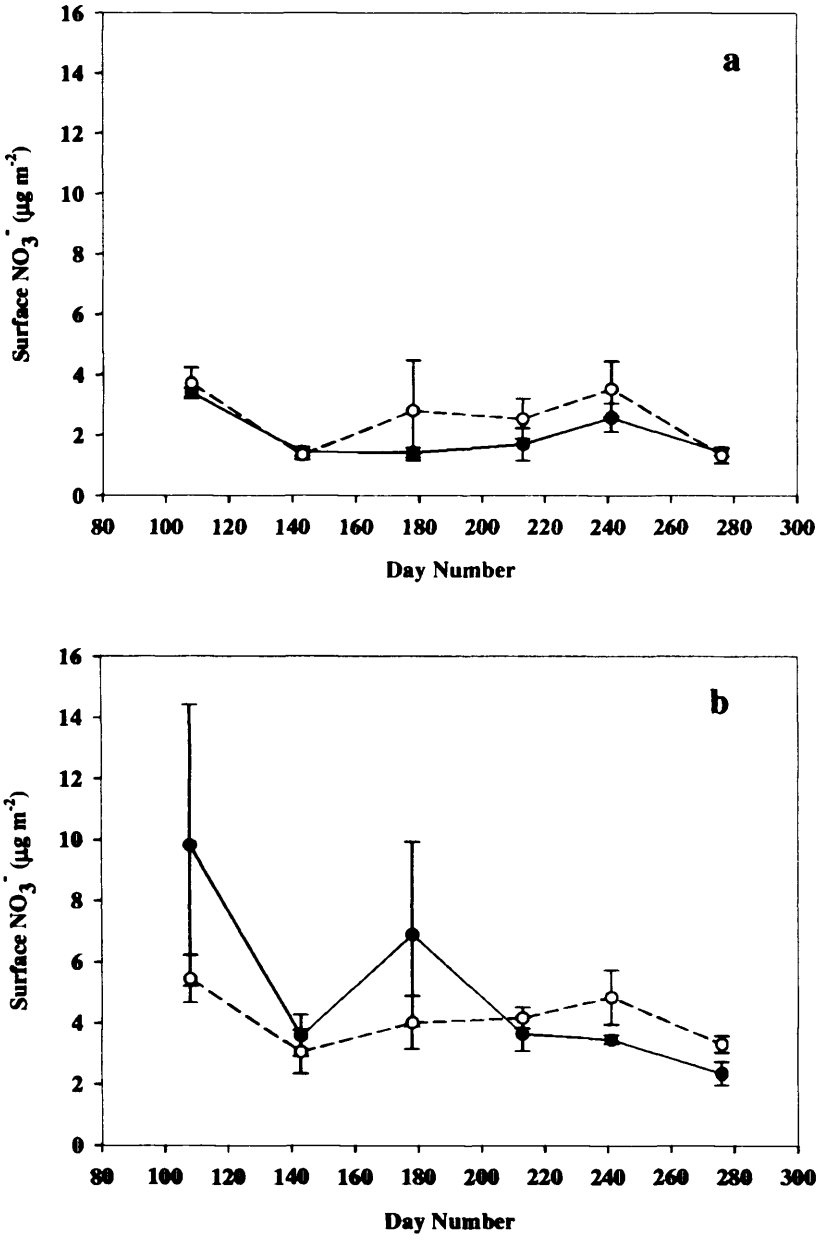


Figure 6.1. Leaf surface NO_3^- amounts on (a) *S. nigra.* and (b) *C. monogyna* at the motorway (solid line) and the control site (dashed line) for 2002. Sampling commenced on 18/04/02 and ended on 03/10/02. Mean values ± SE error bars are shown with the sample size n= 3.

This lack of differences may be explained by the fact that while Chiwa *et al.* (2003) compared spatially very different sites on either side of a mountain where the control represented a clean air site, this study compared sites only a few hundred metres apart and as results in Section 3.2.1. showed the control site is far from pollutant free, receiving approximately 19.6 Kg N ha^{-1} yr^{-1}. It is possible that NO_3^- is only able to accumulate on surfaces up to a threshold amount after which no more NO_3^- is retained. It is proposed that at both sites within Mardley Heath this threshold is saturated by the background NO_x deposition thus preventing us from detecting any differences with respect to surface NO_3^-. There were differences however between the amounts of surface NO_3^- on the two species tested. This may be related to differences in surface characteristics.

6.2.2. Surface Zn

Surface Zn amounts on *S. nigra* and *C. monogyna* leaves were also measured throughout the growing season of 2002 (Figure 6.2.) Just as with surface NO_3^-, amounts of surface Zn appear higher on *C. monogyna* than on *S. nigra* leaves. Amounts range from 0.09 – 3.09 mg Zn m^{-2} for *S. nigra* and from 0.33 – 6.62 mg Zn m^{-2} for *C. monogyna*. These values are approximately 1000 times higher than the amounts of surface NO_3^- suggesting that these two ions are not co-deposited.

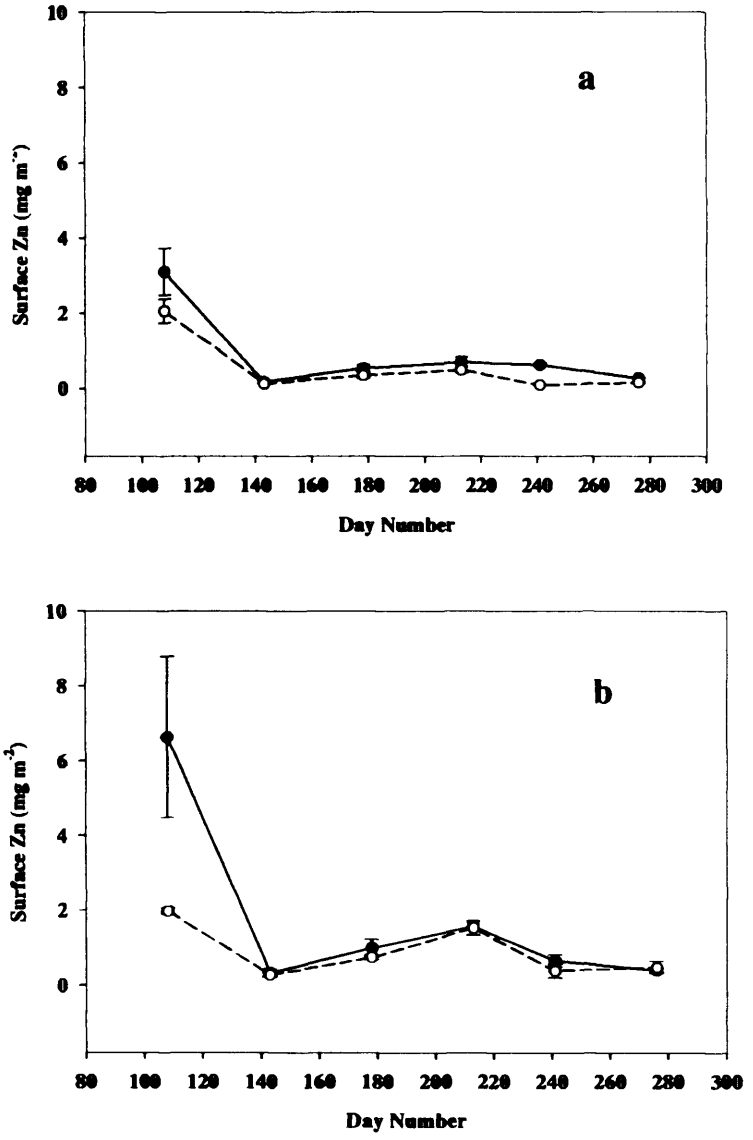


Figure 6.2. Leaf surface Zn amounts on (a) *S. nigra* and (b) *C. monogyna* at the motorway (solid line) and the control site (dashed line) for 2002. Sampling commenced on 18/04/02 and ended on 03/10/02. Mean values ± SE error bars are shown with the sample size n= 3.

The difference between the species is most pronounced early in the year when Zn concentrations are at their peak. This suggests that the lifespan of leaves is unimportant as Zn does not appear to be accumulating over the growing season. These differences between species with respect to surface Zn were confirmed by experiments testing a range of roadside plants collected from Marylebone Road, which is one of the busiest and most polluted roads in London.

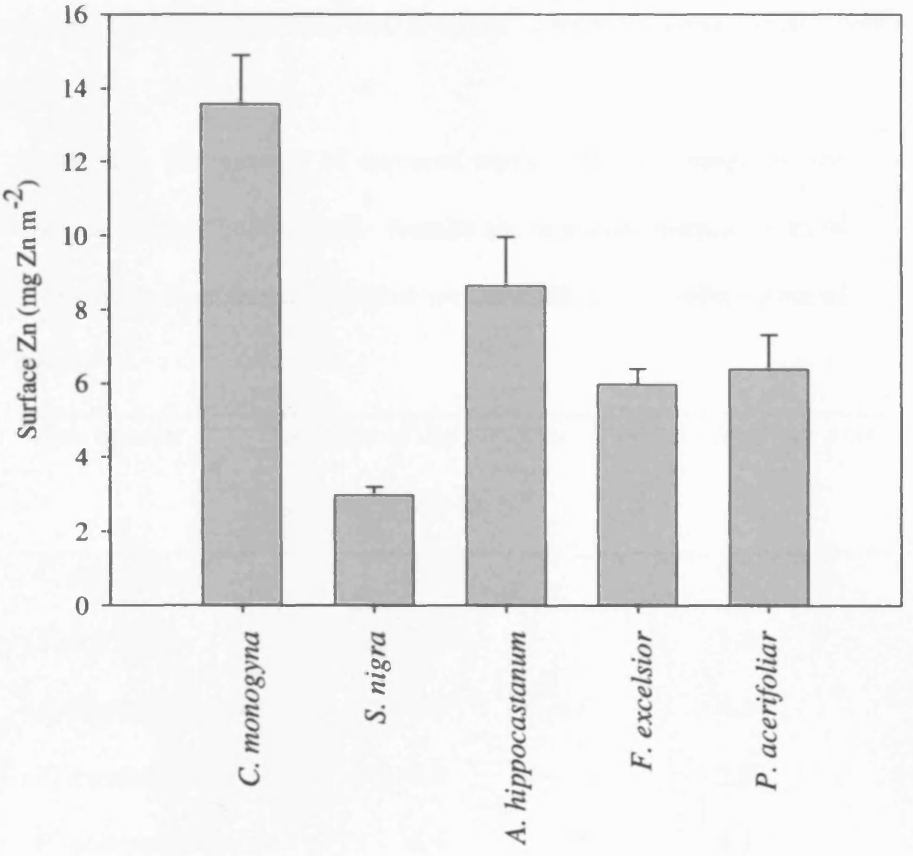


Figure 6.3. Leaf surface Zn amounts on a range of tree species growing in central London. Sampling took place on 08/08/03. Mean values ± SE bars are shown with the sample size n= 3.

A bar chart showing the surface amounts of Zn on leaves of *S. nigra*, *C. monogyna*, *Aesculus hippocastanum*, *Fraxinus excelsior* and *Platanus acerifolia* is shown in Figure 6.3. Once again surface amounts are highest on *C. monogyna* leaves.

Table 6.2 provides estimates of the total Zn load per tree. It should be noted that these figures may be an overestimation of the actual load since they are based on the leaf surface area of a Plane tree of 482 m^{-2} quoted by Beckett *et al.* (1998).

Table 6.2. The amount of captured surface Zn by a range of tree species at Marylebone Road. Results are based on measurements of surface Zn from leaves sampled on 08/08/03 and a surface area of 482 m^{-2}.

Tree Species	*Amount of Zn per area of foliage (mg Zn m^{-2})*	*Total Zn load per tree (g Zn)*
C. monogyna	13.8	6.7
S. nigra	3.0	1.4
A. hippocastanum	8.6	4.1
F. excelsior	6.0	2.9
P. acerifolia	6.4	3.1

The table however does provide a useful insight into the potential total amounts of Zn per tree in an urban environment. Total Zn loads per tree appear to be very

large. From Table 6.2. it appears that *C. monogyna* is able to trap the most surface Zn per tree however in reality the total Zn load per tree may be highest for *P. acerifolia* (London Plane) which has a larger surface area that is likely to be similar to the surface area used for the calculation of figures in Table 6.2.

Deposition of particles to vegetation is influenced by the frictional drag of the vegetation. Forests have a larger frictional drag than grasslands and thus receive more particulate deposition. The same theory applies on a smaller scale at the plant cuticle (Beckett *et al.*, 1998). Cuticle roughness can vary between species due to differences in microstructural ornamentation such as epicuticular wax. Wax is present both within the cuticle and on the cuticle surface. It is this surface wax that is referred to as epicuticular wax. Depending on its chemical composition, epicuticular wax is able to form crystals that cover the surface in a regular microrelief of about 1-5 μm in height (Jeffree, 1986). In order to determine whether the observed differences in particle retention could be related to differences in the epicuticular waxes a scanning electron microscope was used to look at leaf surfaces in greater detail. Scanning electron micrographs of *S. nigra* and *C. monogyna* leaves are presented in Figures 6.4, 6.5, 6.6 and 6.7. Only Figure 6.4 shows the upper epidermal surfaces, the remainder show the lower surfaces as on the occasions when the upper epidermal surface was observed difficulties with the scanning electron microscope always seemed to occur and as a result fewer micrographs were obtained.

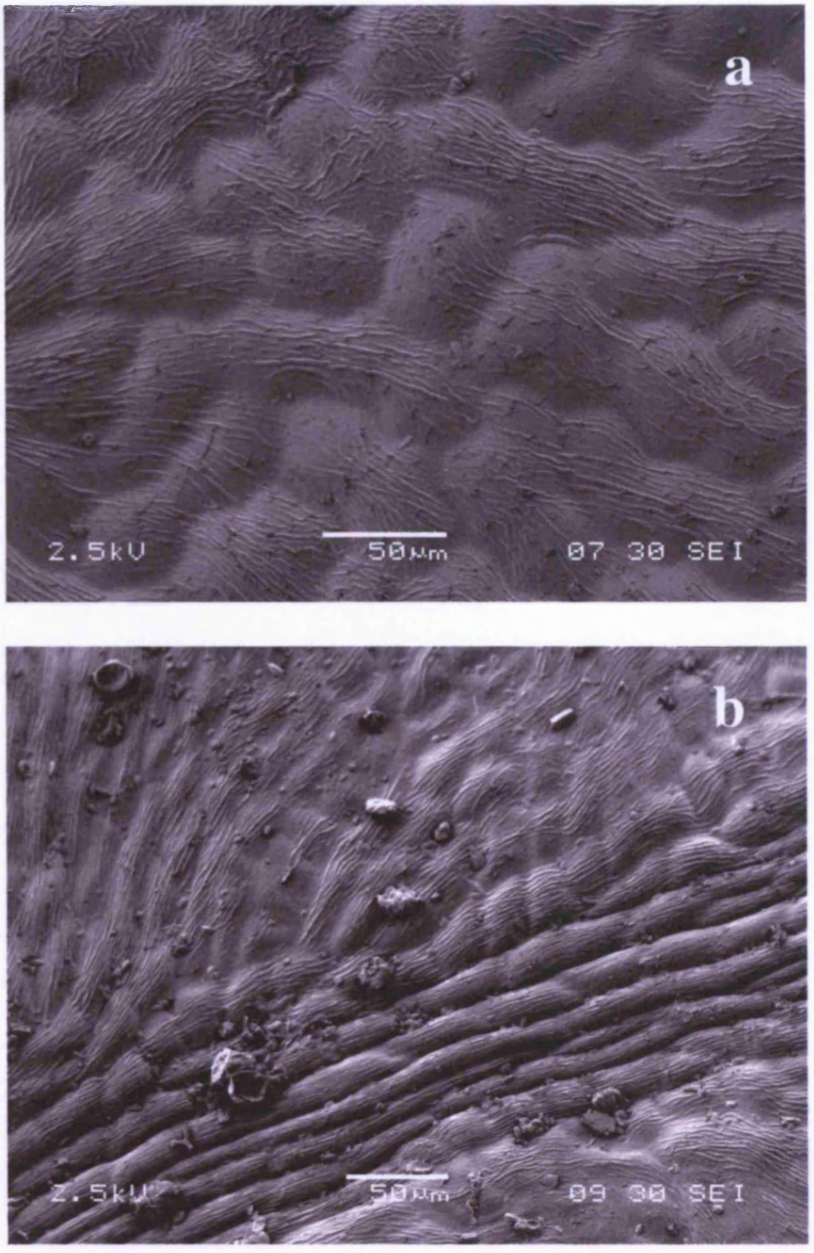


Figure 6.4. Scanning electron micrographs of the upper epidermal surface of (a) *S. nigra* and (b) *C. monogyna*. Leaves were sampled from Marylebone Road on 24.06.03. The white bar = 50 µm

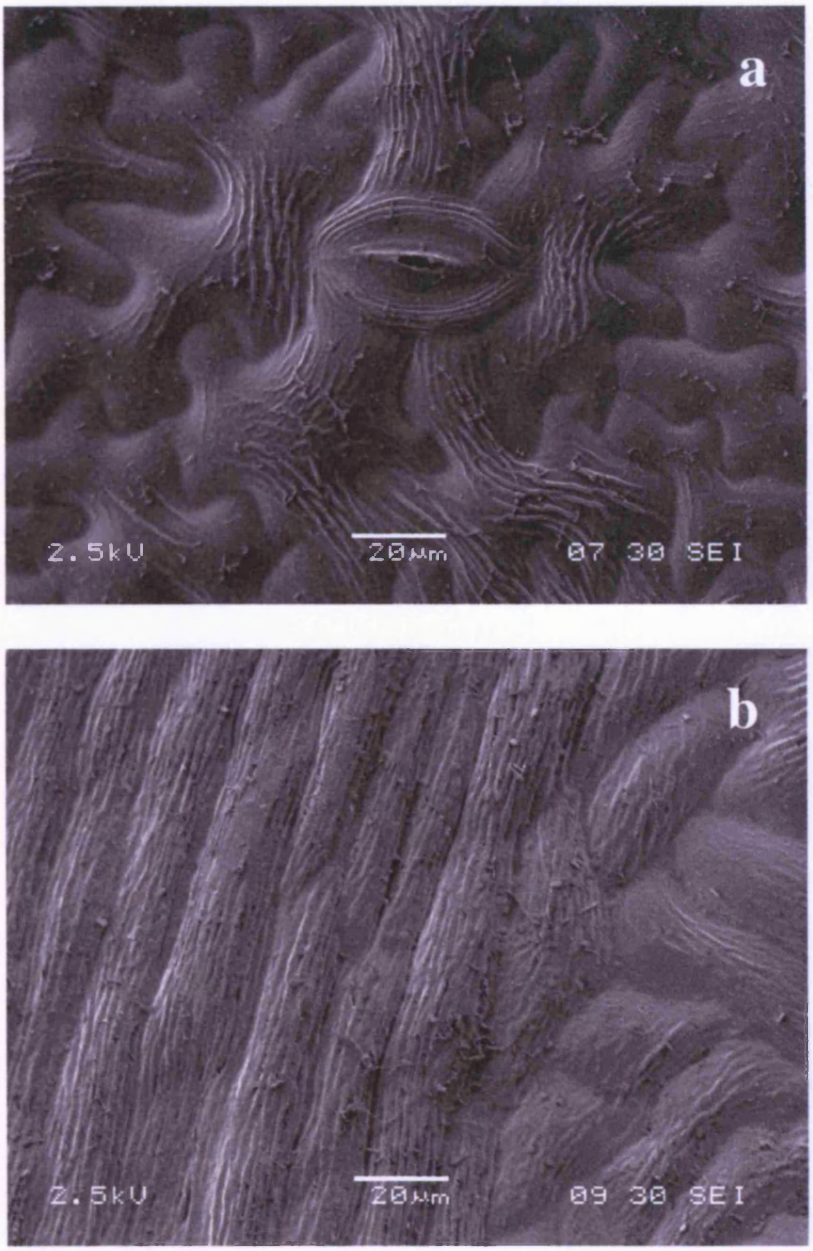


Figure 6.5. Scanning electron micrographs of the lower epidermal surface of (a) *S. nigra* and (b) *C. monogyna*. Leaves were sampled from Marylebone Road on 24.06.03. The white bar = 20 µm

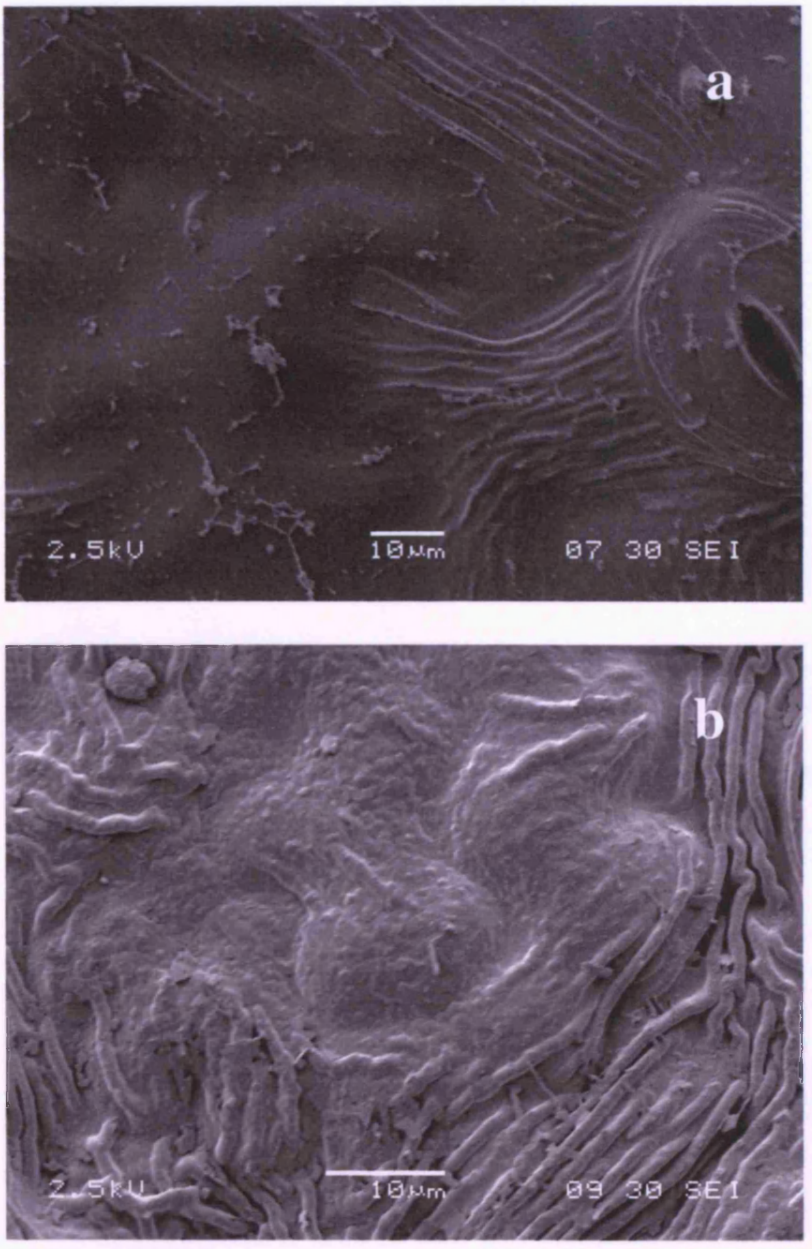


Figure 6.6. Scanning electron micrographs of the lower epidermal surface of (a) *S. nigra* and (b) *C. monogyna*. Leaves were sampled from Marylebone Road on 24.06.03. The white bar = 10 µm

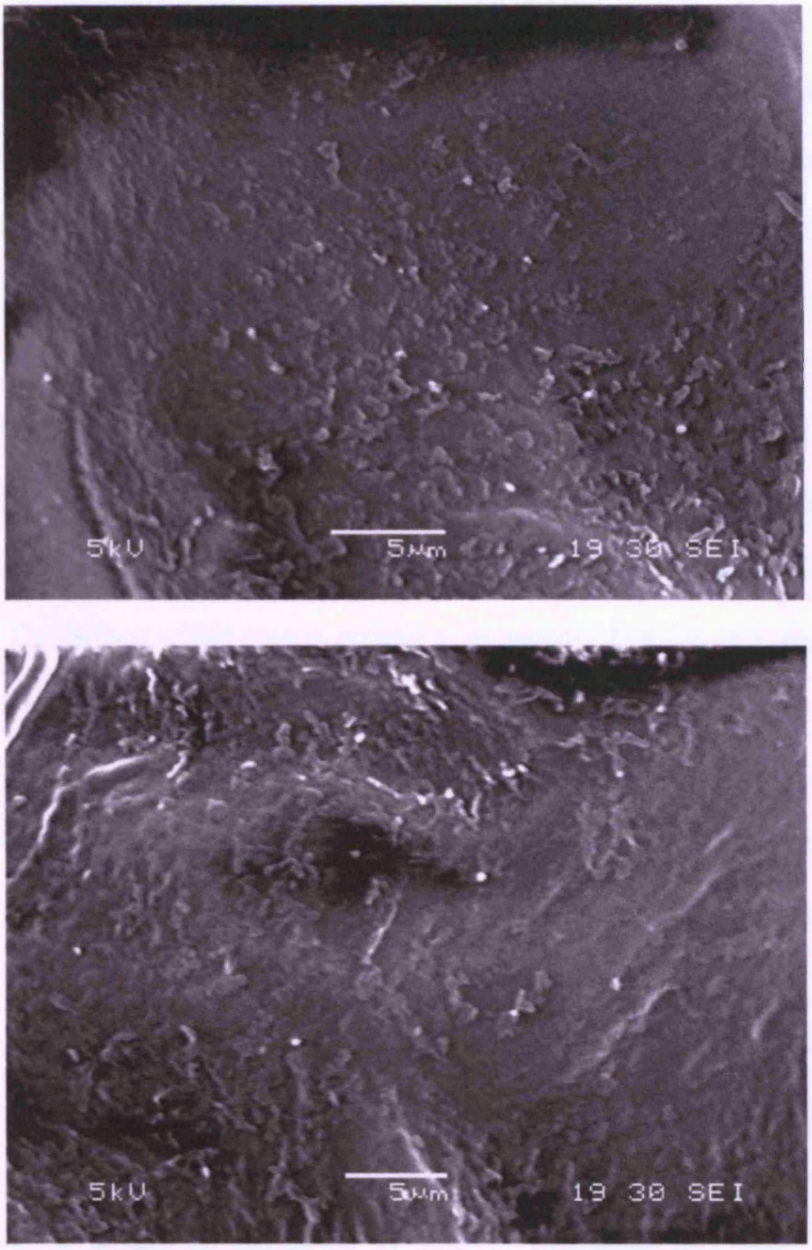


Figure 6.7. Scanning electron micrographs of the lower epidermal surface of *C. monogyna*. Leaves were sampled from Marylebone Road on 24.06.03. The white bar = 5 µm

The convex epidermal cells of both species can clearly be seen in the micrographs. At lower magnification (Figure 6.4) no differences in epidermal microstructure can be observed. Both species appear to have cuticular folds. Figure 6.4 also shows the large amount of particle contamination present on these leaves and a leaf vein is observable in Figure 6.4.b.

At higher magnification the cuticular folds are still observable in *S. nigra* although portions of the cells appear smooth (Figure 6.5.a). The micrograph of *C. monogyna* shows part of a leaf vein and some adjacent epidermal cells (Figure 6.5.b). The surface of this species appears to be rougher than that of *S. nigra* and has a string-like coating

In Figure 6.6 differences between the species are more obvious. *S. nigra* (Figure 6.6.a) leaves appear almost completely lacking in microstructures whereas *C. monogyna* (Figure 6.6.b) still has the same rough, string-like appearance. This may in fact be epicuticular wax crystals. Wax crystals can take the form of filaments, plates, tubes or spirals. The crystals observable on *C. monogyna* may be wax tubules. In certain areas these tubules appear to have been transformed into platelets as well as coalescing to form an amorphous wax layer. Such degradation occurs as the result of natural senecesence (Neinhuis and Barthlott, 1998) although the process may be accelerated by exposure to pollutants such as NO_2 which cause oxidative damage to wax components (Jetter *et al.*, 1996). Figure 6.7. shows the epicuticular wax on *C. monogyna* leaves at a higher magnification. The surface appears to have a slightly granular appearance which may represent degraded tubules.

It seems likely that these differences in epicuticular wax structures may in part explain the higher amounts of surface Zn and NO_3^- observed on *C. monogyna*, compared to *S. nigra*. However, while rough surfaces, such as those of *C. monogyna* have a greater capacity to trap airborne particulates, this is compensated for by a very effective self-cleaning capability of rough leaves (Barthlott and Neinhuis, 1997). This was first observed during the routine observations of leaf surfaces using SEM when it was found that smooth leaf surfaces always had to be cleaned prior to analysis while those exhibiting a high degree of wax ornamentation were almost completely free of contamination (Barthlott, 1990).

Rainfall during 2002 was considered in conjunction with the surface Zn data to see if this had any involvement in the accumulation of surface Zn. The relationship between surface Zn and rainfall is shown in Figure 6.9. Linear relationships were found between deposition amounts of Zn on unit surface area of *S. nigra* and *C. monogyna* and the amount of precipitation in the previous 25 days.

It appears that although Zn may accumulate on leaves during dry periods it is easily washed off the leaves by rainfall events. The self-cleaning properties of leaves is known as the 'lotus-effect' after the large peltate leaves of the sacred lotus, *Nelumbo nucifera* (Barthlott and Neinhuis, 1997).

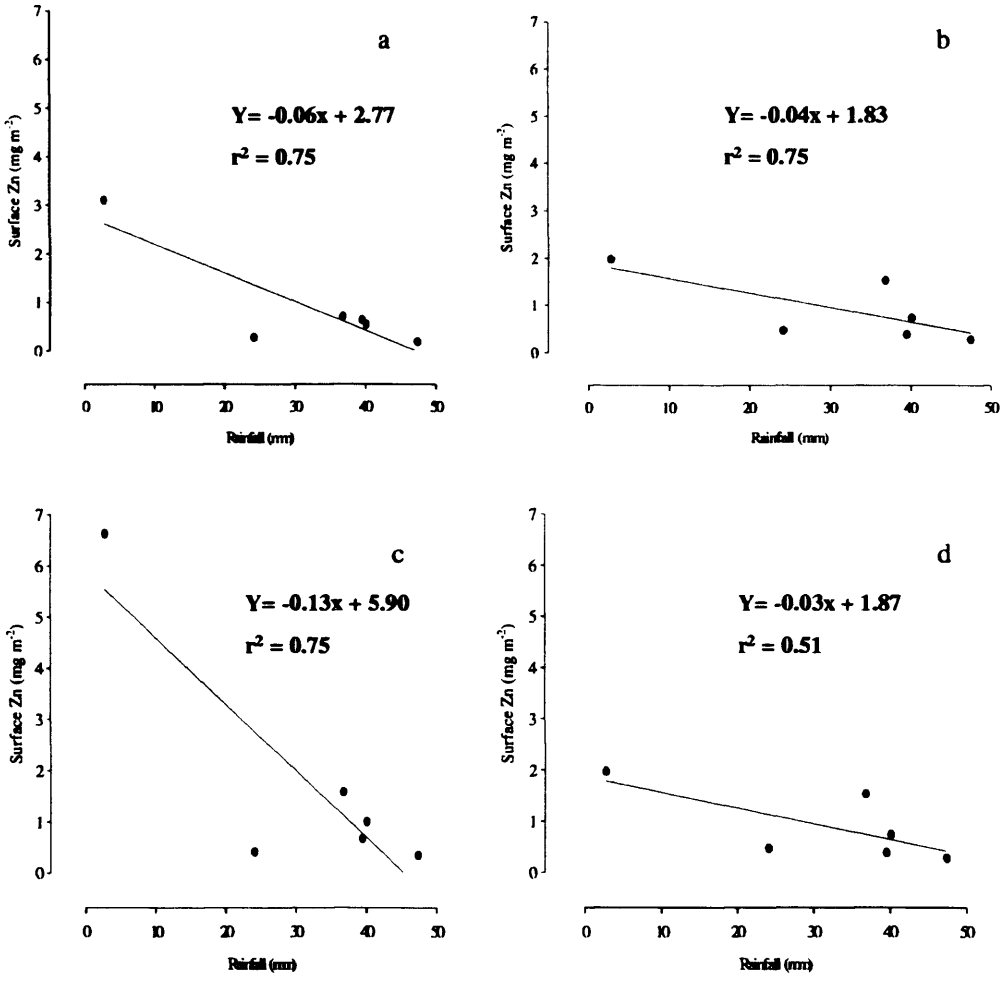


Figure 6.9. Relationship between amounts of surface Zn and rainfall in the 25 days preceding sampling for (a) *S. nigra* at the motorway site (b) *S. nigra* at the control site (c) *C. monogyna* at the motorway site and (d) *C. monogyna* at the control site.

The lotus effect may play an important role in the defence against pathogens. Spores and conidia of pathogenic microorganisms will be removed by rainfall on leaves with a high degree of wax ornamentation. Damage to the wax layer, for example pollution damage, can erode this layer and reduce the ability of leaves to clean themselves

The lotus-effect is largely due to a lower particle adhesion to rough surfaces than to smooth surfaces. On rough surfaces particles tend to be larger than the surface microstructures and therefore rest only on the very tips of the wax crystals and thus have less surface area contact with the cuticle. In addition, high contact angles on rough surfaces mean that rainfall forms spherical droplets that remove particles as they role off the leaf with a high velocity. On smooth surfaces however, water will form droplets with low contact angles and low velocities. Rather than removing particles from the leaf surface, these droplets tend to redistribute any particulates (Barthlott and Neinhuis, 1997). This self-cleaning ability of leaves is less apparent when precipitation occurs as fog and dew instead of rain. Rain droplets have high kinetic energy that is necessary to remove deposited particles. Heavy rain during thunderstorms may by especially effective at cleaning leaves (Barthlott and Neinhuis, 1997).

In this research it appears that *C. monogyna* receives the most Zn deposition during dry periods. This may be the result of epicuticular wax crystals present on the cuticle which were observed using the scanning electron microscope (Figure 6.4 to 6.7). During rainfall events however, Zn is not retained on the surface and is washed off the leaves (Figure 6.9). *C. monogyna* leaves may be particularly

effective at 'self-cleaning' due to enhanced surface roughness. Indeed, amounts of surface Zn show a 20 fold decrease following the rainfall events that occurred between the first two sampling dates in 2002.

The surface Zn measured on trees at Mardley Heath (Figure 6.2) may originate from either (i) background atmospheric Zn as this can range from 0.036 to 63 ng m^{-3} in semi-rural and rural locations (Baker, 1997; Allen et al., 2001) or (ii) from traffic using the nearby motorway. According to Harrison (2003) Zn particles are evenly distributed through the size fractions. Therefore while course particles may be deposited close to the motorway, fine particles can travel further and may affect surface amounts at the control site.

Although road traffic is a source of Zn (Harrison, 2003) no significant differences were observed between surface Zn at the motorway and at the control sites for either species (Figure 6.2.). Early in the season surface amounts of Zn were higher and for *C. monogyna* there appeared to be a difference, albeit insignificant, between the sites. As previously discussed, the low amounts of rainfall may have permitted surface Zn accumulation to occur at the motorway site to a greater extent than at the control site. Rainfall events throughout the remainder of the year may have prevented the build up of surface Zn at the motorway site. Although leaf surface Zn may be affected by traffic flow, it does not seem a suitable biomarker due to the high efficiency with which leaves appear to be able to cleanse themselves during rainfall.

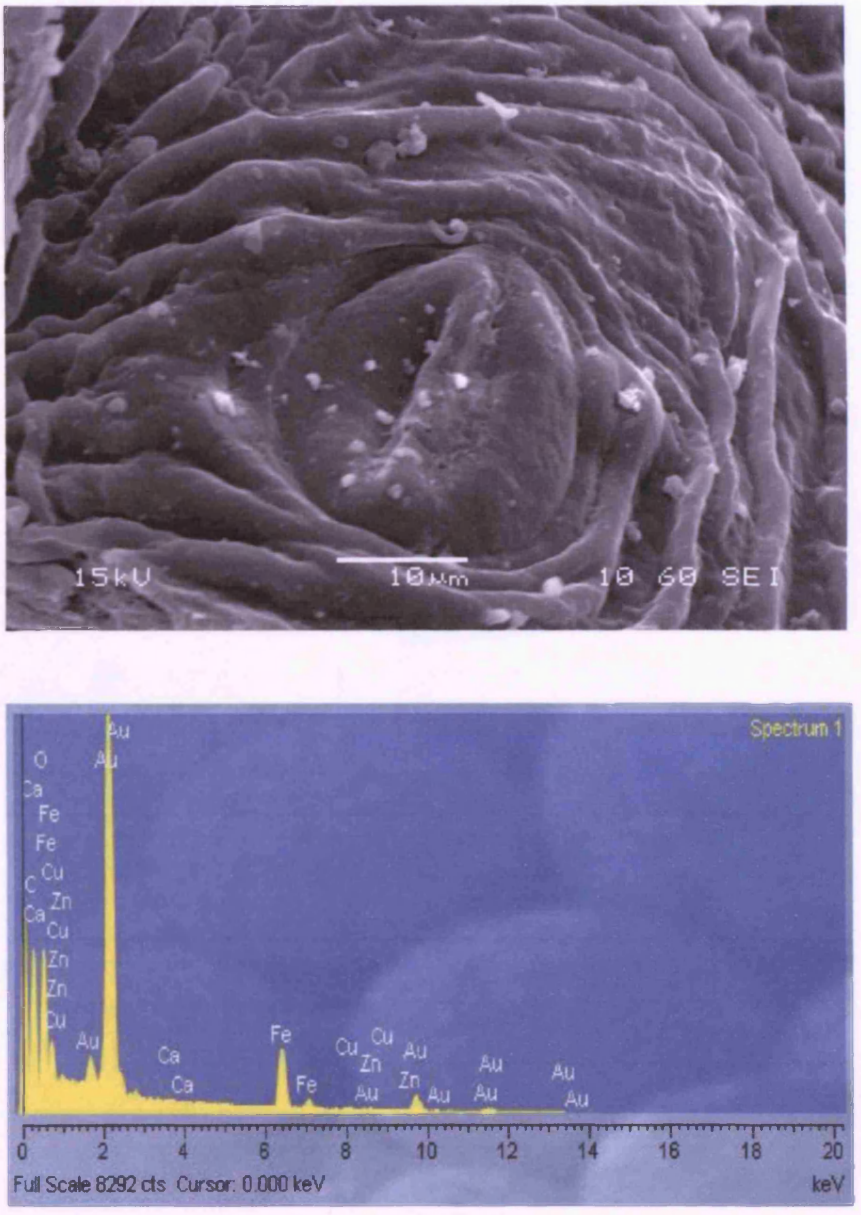


Figure 6.10 *C. monogyna* lower epidermis EDS microanalysis.

Leaf material was sampled from Marylebone Road on 01.07.03.

Upper image: Scan area. The white bar = 10 µm

Lower image: Resulting emission spectra.

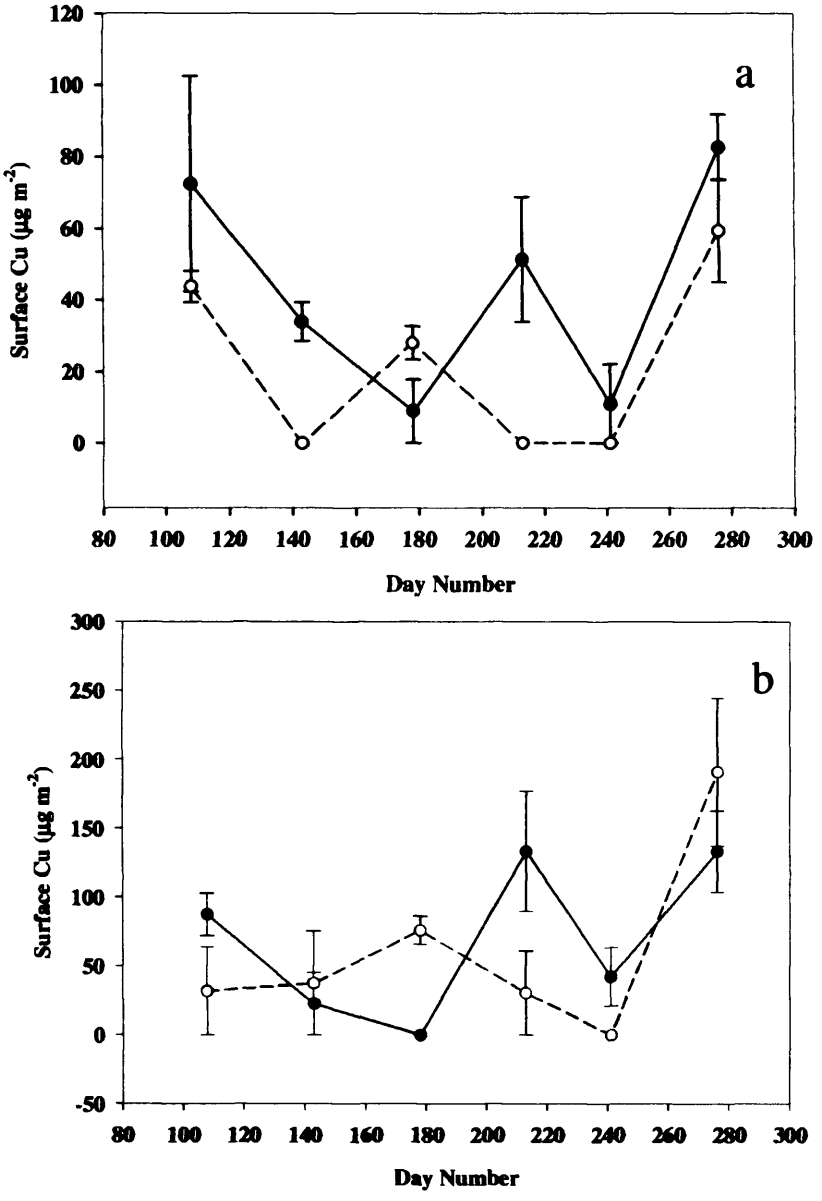


Figure 6.11. Leaf surface Cu amounts on (a) *S. nigra* and (b) *C. monogyna* at the motorway (solid line) and the control site (dashed line) for 2002. Sampling commenced on 18/04/02 and ended on 03/10/02. Mean values ± SE error bars are shown with the sample size n= 3.

Scanning electron microscopy in conjunction with energy dispersive microanalysis (EDS) was employed to investigate the characteristics of surface Zn particulates further. This technique is based upon the measurement of characteristic X-rays emitted from the sample during scanning electron microscopy which allow the elemental composition of the sample to be determined. Zn was detected on the surfaces of both *S. nigra* and *C. monogyna* leaves collected from Marylebone Road. Figure 6.10 shows one example of a scan of *C. Monogyna*. Wax tubules, a stomata and numerous surface particulates can be seen in the scan area (Figure 6.10, upper image). The EDS microanalysis emission spectra for the scan area is shown in Figure 6.10 (lower image). The elements Fe, Cu and Zn were all detected in this scan area.

The aim of using EDS was to characterise surface Zn particles according to size and shape. Unfortunately, although this technique did show the presence of Zn within the scan area on several occasions, Zn could not be pinpointed to any particles observable on the leaf. This may explained by Zn being present on surfaces as very fine particles or because amounts of surface Zn were below the detection capabilities of the EDS equipment. Time restrictions when for using the scanning electron microscope and EDS equipment meant that this topic was not investigated further.

Use of EDS microanalysis allowed the detection of a range of other surface heavy metals most commonly, Cu and Fe. Fe-containing particles are most likely to originate from wind-blown soil dusts. Cu may originate from road traffic since this element has previously been found at roadsides (Harrison, 2003). Following

the detection of surface Cu using EDS microanalysis, the leaf Zn leaf wash solutions were re-examined for Cu content (Figure 6.11). Surface amounts of Cu were much lower than amounts of surface Zn suggesting that Cu particles are emitted from traffic in smaller amounts than Zn-containing particles and are therefore this element is unlikely to be a useful biomarker for traffic exposure.

6.2.3. Tissue Zn

As discussed in Section 6.1.5. root uptake of Zn can result in phytotoxic effects on plants. Phytotoxic effects could also occur if Zn particles were to enter leaves directly through the stomata. As this thesis is mainly interested in deposition and assimilation of N the effects of Zn on the NR activity of *S. nigra* leaves was investigated using an *in vitro* assay with Zn additions. Previous work has shown the inhibition of NR activity by Zn (Luna *et al.*, 2000). The results of Zn on *in vitro* NR activity in *S. nigra* are presented in Figure 6.12. Results show that inhibition of *S. nigra in vitro* NR activity by Zn occur with 500 μM Zn additions to the assay medium. A paired T-test revealed this difference to be significant at the 5% level ($p=0.029$). The mechanism of NR inhibition may involve Zn causing oxidative damage resulting in the inefficient transfer of electrons within the enzyme complex (Smarrelli and Cambell, 1983) rather than the reactions with NR sulphydryl groups as was previously hypothesised (Luna *et al.*, 1997, Luna *et al.*, 2000).

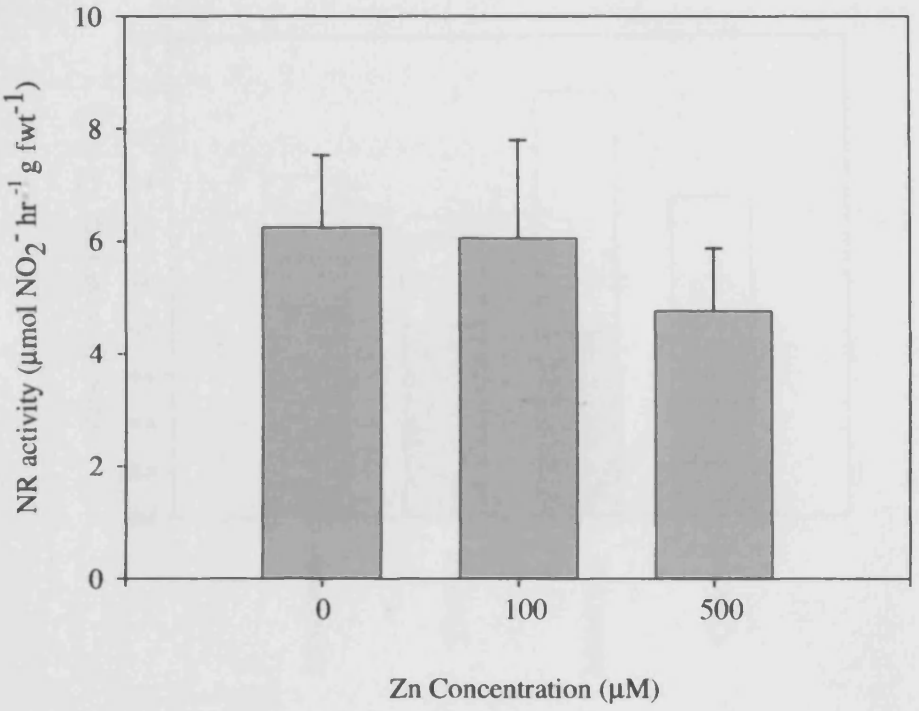


Figure 6.12. Effect of Zn additions to the *in vitro* assay medium on NR activity of greenhouse grown *S. nigra* leaves. Four *S. nigra* plants were used to perform one each of the 3 assays. Mean values ± SE bars are shown with the sample size n=4.

Whether Zn is affecting foliar NR in the field will depend on whether Zn particles are sufficiently small to pass through stomata. Zn particles in the roadside environment range in size from < 0.2 µm to > 10 µm aerodynamic diameter (Harrison, 2003).

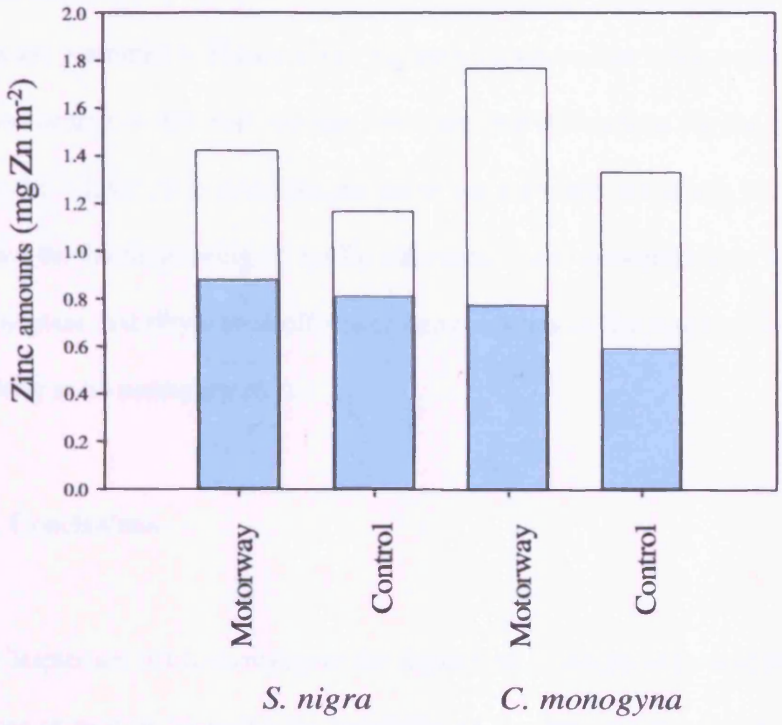


Figure 6.13. Tissue and surface Zn amounts for *S. nigra* and *C. monogyna* leaves. Leaf material was collected at Mardley Heath on 27/06/02. The lower, blue portion of the bar represents the amount of Zn in the leaf tissue whereas the top, white portion of the graph represents the surface Zn removed by 0.01 M HCl wash.

Therefore the finer Zn fractions may be able to enter leaves via the stomata which usually measure 8-10 μm in diameter (Farmer, 2002). Concentrations of Zn in leaves are presented in Figure 6.13. Figure 6.13 shows that while surface amounts vary according to site and species, there are few differences for the amounts of tissue Zn. Leaf Zn concentrations alone are therefore not likely to be a useful tool for the biomonitoring of traffic exposure. Low concentrations of foliar Zn also indicate that phytotoxic effects of heavy metals as the result of foliar uptake are likely to be unimportant.

6.2.4. Conclusions

This Chapter set out to investigate the deposition of Zn particles and NO_3^- to leaf surfaces as well as assessing the suitability of 3 techniques for monitoring traffic flow namely, measurements of surface NO_3^-, surface Zn and leaf Zn.

With regards to surface NO_3^- no significant differences were found between leaves collected at the motorway site and at the control site. As mentioned previously in this Chapter, it is hypothesised that the reason we do not observe significant differences between the two sites is because the surface NO_3^- retention capacity is exceeded at both the motorway and the control sites, due to the large amount of background NO_x deposition at Mardley Heath. Since other workers have been able to demonstrate differences in surface NO_3^- (Bytnerowicz *et al.*, 1991; Sanz *et al.*, 2002; Chiwa *et al.*, 2003) it is proposed that measurements of surface NO_3^- amounts, although not useful as biomarkers at Mardley Heath, may be useful at sites receiving low levels of deposition.

Measurements of leaf tissue Zn are not recommended as techniques for biomonitoring as this did not show significant differences between the two pollution sites suggesting that Zn particles were largely unable to enter leaves. Surface Zn was highly influenced by rainfall events which prevented the accumulation of Zn at the motorway site. This close relationship with rainfall indicates that surface Zn amounts also cannot be used as a reliable biomarker. Perhaps outside of the UK, in a country with a drier climate, surface Zn may offer more potential as a biomonitoring tool.

Differences in amounts of surface Zn and NO_3^- between the species was investigated using scanning electron microscopy techniques which revealed a smooth cuticle on the leaves of *S. nigra* and a cuticle to be covered with tubular wax epicuticular crystals on the leaves of *C. monogyna*. The observed differences in surface Zn particulates and NO_3^- amounts are therefore attributed to the fact that *C. monogyna* has a rougher surface which increases particulate deposition due to the greater frictional drag of this surface on air-flow. When a range of species growing in central London were analysed, surface Zn was found to be highest on *C. monogyna* leaves. Total amounts of *C. monogyna* per tree were estimated to be in the region of 6.7 g Zn. This indicates the large capacity of this species to capture large amounts of Zn and likely other harmful particulates. As the results have demonstrated, this surface Zn is likely to be transferred to the soil via stem flow during rainfall events. Therefore the amount of Zn trapped by trees over the entire growing season is likely to be several times this value. Since traffic particulates are estimated to contribute to more human deaths annually than road accidents (WHO, 1999) this ability of trees to trap particulates and channel them

to the soil, suggests that the planting of certain trees in heavily polluted environments may be a useful tool for improving both pollution levels and human health.

CHAPTER 7

GENERAL DISCUSSION

7.1. Introduction

As stated in Section 1.9, this work had three main objectives:

1. To assess the contribution of traffic-derived atmospheric NO_x to N deposition at a deciduous woodland adjacent to a motorway and to establish whether this deposition was having a fertilising effect on plant growth.
2. To investigate the effects of increased N deposition on foliar uptake and tree physiology.
3. To investigate the deposition of Zn particles and NO_3^- to leaf surfaces as techniques for monitoring traffic exposure.

This Chapter assesses the achievement of these aims and discusses the results in relation to potential biomarkers for N deposition and traffic exposure as well as the use of trees for improving air quality near pollution sources.

7.2. Contribution of traffic-derived NOx to N deposition and tree growth.

Deposition of NO_x within the woodland at Mardley Heath ranged from 14.8 to 40.7 Kg N ha^{-1} yr^{-1}. These values exceed the current woodland critical loads of 10-20 Kg N ha^{-1} yr^{-1} (Achermann and Bobbink, 2003) as well as the estimated total annual requirement for trees of 8-12 Kg N ha^{-1} yr^{-1} (Jenkinson, 1971; Pearson and Stewart, 1993). Deposition is however, less than half that of the estimated N deposition to Rothamsted woodland, approximately 20 miles away in the neighbouring county, Hertfordshire (Goulding *et al.*, 1998) which has been predicted to be in the region of 100 kg ha^{-1} yr^{-1} suggesting that this is perhaps a large overestimation.

NO_x deposition within Mardley Heath is spatially related to distance from the motorway reaching a maximum in nearby the rural downwind environment. There appeared to be a clear difference between the amount of NO_x deposition occurring at the two sites where the majority of sampling was undertaken (the motorway and the control sites). This difference in pollution exposure along the transect is assumed to be a major factor when considering vegetation response at the two sites. If it is assumed that deposition at the control site was not influenced by traffic inputs and is simply representative of typical background UK NO_x levels it is possible to estimate the contribution of traffic derived N to trees at the motorway site at 25.9 Kg N ha^{-1} yr^{-1} (175 % higher than the control site).

N is an essential nutrient that often limits plant growth. In Section 3.2.4. it was shown that growth, as measured by shoot extension was significantly higher in

both *S. nigra* (p=0.003) and *C. monogyna* (p=0.027) at the motorway site relative to the control. Although other possible variables between the sites such as temperature, CO_2 and light availability may have influenced this result, increased N availability as the result of deposition of traffic-derived N at the motorway site is likely to be a major contributory factor since the fertilizing effects of N deposition on trees are well documented (Kauppi *et al.*, 1992; Nasholm, 1998; Spiecker, 1999).

S. nigra appears to responds more strongly than *C. monogyna.* This may be because as a nitrophilous pioneer, *S. nigra* has a greater capacity for N uptake and assimilation than the less nitrophilous *C. monogyna.* According to Nilsson (1986), a positive growth response to N additions indicates that the habitat is not N saturated therefore we can conclude that Mardley Heath is not N saturated and N is limiting growth at the control site.

7.2.1. Achievement of objective

This study measured dry deposition at Mardley Heath in the form of NO_2 using diffusion tubes. Deposition of NO_x was closely related to distance from the motorway, providing strong evidence that this is a point source of atmospheric NO_x. Tree growth of *S. nigra* and *C. monogyna* in the downwind environment was assessed by measuring shoot extension and number of leaf nodes towards the end of the growing season. Growth, in terms of shoot extension was significantly higher in trees at the motorway site relative to the control. The combination of increased NO_x deposition and increased shoot extension at the motorway site

relative to the control was taken to suggest that traffic-derived N was having a fertilizing effect on trees. The first objective of this study was thus fully met.

7.3. Effect of traffic-derived N on foliar N uptake and tree physiology

Further evidence for the contribution of traffic-derived N to plant nutrition was provided by the $\delta^{15}N$ work (Section 3.2.2). The $\delta^{15}N$ signature of tree foliage (*C. monogyna* and *Quercus spp.*) became less negative close to the motorway which showed that traffic-derived N was being taken up and assimilated by the trees. Additionally, these results support several other studies that have reported a positive $\delta^{15}N$ signature for road traffic emissions. Although these results showed that motorway derived N was entering roadside trees, the route of entry i.e. whether N was taken up directly via the leaves or whether it is first deposited to the soil and taken up by the roots remained unclear. Thus measurements of the activity of the foliar N assimilatory enzyme, NR were carried out in order to help determine the entry route.

Activity of NR was measured in four species, *C. monogyna*, *S. nigra*, *B. pubescens* and *Quercus spp.* The only species that showed significantly higher foliar assimilation of NO_3^- at the motorway site relative to the control was *S. nigra* ($p=0.020$) suggesting that this species may be able to take up atmospheric NO_x directly via the stomata. The high NR activity of this nitrophilous pioneer may create a sink for atmospheric NO_x effectively allowing the plant to 'mop up' NO_x

from the atmosphere. The stimulated growth of this species at the motorway (Section 3.2.4) suggests the products of NR enzyme are diverted to the production of new biomass. For the other species, foliar assimilation by NR is not significantly higher in individuals at the motorway site.

Therefore in *C. monogyna* and *Quercus spp.* for which the $\delta^{15}N$ data suggested assimilation of traffic-derived N was occurring, uptake of N must be occurring predominantly via the roots. Attempts to investigate root to shoot transfer of N compounds by measuring concentrations of NO_3^-, NH_4^+ and amino acids in the xylem found no significant differences between the motorway and the control sites for either *S. nigra* or *C. monogyna* (See Chapter 4). Although, it should be noted that xylem concentrations do not represent actual flux to the leaves since transpiration rate was not taken into account.

In *C. monogyna* there are some differences in leaf tissue amino acids between the two sites (see Section 4.2.4). From May onwards the tissue concentrations in individuals at the control site are seen to increase at a higher rate than those at the motorway site. A paired T-test revealed that this difference was significant at the 5% level between sampling days 178 and day 276 ($p= 0.034$). Increases in tissue amino acid concentrations are often associated with preparation for senescence as cellular proteins are broken down into their constituent components in preparation for export from the leaf prior to leaf fall. Increases in amino acids at the control site occurring earlier in the year than at the motorway site may indicate perhaps that senescence is somehow being delayed by traffic-pollution. The data showing

tissue sugar concentrations in *C. monogyna* (Figure 4.7.b), although not significant, seem also to indicate a different response between the two sites at senescence. Environmental factors at the motorway site such as elevated CO_2, or elevated N deposition could be responsible for delayed senescence. N deposition may delay senescence through by stimulation of the plant hormone cytokinin (Collier *et al.*, 2003) as increased supply of this hormone supposedly delays the onset of senescence (Marschner, 1995). It may be useful for future studies to examine the levels of this plant hormone in samples taken from the two sites.

The effect of N deposition on total leaf N was investigated. Previous work has linked increases in total N with increased N exposure and thus proposed that measurements of total N may offer a useful biomonitoring method (Port and Thompson, 1980; Baddeley *et al*, 1994; Pitcairn *et al.*, 1995; Woolgrove and Woodin, 1996; Hogberg *et al.*, 1998; Pitcairn *et al.*, 2001; Kirkham, 2001). Despite a possible trend for elevated total N per g dwt leaf material at the motorway site relative to the control for *S. nigra*, there are no statistically significant differences for either *S. nigra* or *C. monogyna* regarding total N content. The lack of a significant increase in N may be explained by dilution of the plant N content by increased production of biomass which allows total N per unit of leaf material to remain at a nearly constant level. Indeed the accumulation of tissue N is not believed to occur until the potential for growth has been satisfied, for example when another nutrient becomes limiting (Lee *et al.*, 1986; Zhen and Leigh, 1990).

N deposition has previously been associated with deficiencies in other mineral nutrients, since biomass production may be stimulated beyond nutrient availability. As earlier results have shown significantly higher growth was occurring at the motorway site relative to the control, we may therefore expect to find lower foliar concentrations of mineral nutrients at the motorway. The results in Section 3.2.5 however showed no significant differences between the sites with respect to total P for both *S. nigra* and *C. monogyna.* From this it may be concluded that soil P is not limiting at Mardley Heath and is able to support the stimulated growth that is occurring in trees at the motorway site. In fact high availability of soil P may be a contributing factor to enhanced growth at the motorway site since continued growth as the result of N deposition is only possible if other nutrients are not limiting.

7.3.1. Achievement of objective

The second objective of this work, to investigate the effects of increased N deposition on foliar uptake of N and physiological process was also achieved. While $\delta^{15}N$ data provided evidence of the contribution of traffic-derived N to plant N nutrition, information regarding foliar uptake was inferred from measurements of leaf NR activity. *S. nigra* was the only species that exhibited a significant increase in foliar NR activity at the motorway site relative to the control and therefore foliar uptake is presumed only to be important for this species. Seasonal measurements of tissue and xylem concentrations of NH_4^+, PO_4^{3-} and NO_3^- did not reveal any significant differences between the two sites for

either species (see Chapter 4). However, increases in concentrations of tissue amino acids however were observed to occur earlier in the season at the control site relative to the motorway site for *C. monogyna* perhaps suggesting that the motorway is somehow influencing the phenology of this species. One limitation of this work was that measurements of xylem compounds could not be relied on to provide accurate information for supply to the leaves since transpiration, and therefore flux was not measured. It is therefore recommended that similar future studies measure transpirational flow in conjunction with xylem concentrations in order to gain a better insight into supply of these compounds to the leaves. Total N per unit of leaf material was not seen to increase in trees at the motorway site. Similarly, no significant differences were detected with respect to total P. It is concluded that while N is limiting at the Mardley Heath woodland, P is not, thus allowing the stimulation of growth in individuals at the motorway site. This growth is occurring at a rate which allows the total N and P per unit of leaf material to remain at a constant level.

7.4. The deposition of Zn particulates and NO_3^- to leaf surfaces

Deposition of Zn and NO_3^- was assessed by foliar washing technique. Amounts of surface NO_3^- were small and there were no significant differences between surface NO_3^- at motorway site and at the control site. This contrasts with several recent studies that have suggested that measurements of surface NO_3^- are a useful tool for estimating NO_3^- deposition (Hanson and Lindberg, 1991; Bytnerowicz *et al.*, 1991; Sanz *et al.*, 2002; Chiwa *et al.*, 2003). It is proposed that surface NO_3^-

will only accumulate to a threshold level above which further increases in NO_x deposition will not affect surface NO_3^- amounts. Therefore the reason we do not observe differences in surface NO_3^- amounts between the two sites is because this threshold is exceeded at both the motorway and control sites. It may be the case that measurements of surface NO_3^- amounts are only useful biomarkers for N deposition at sites receiving low levels of deposition.

Surface Zn however did show a tendency to be higher at the motorway site relative to the control however these differences were not significant. What was very apparent in the data was the difference in accumulation between the two species sampled, *C. monogyna* and *S. nigra.* Leaf surfaces of *C. monogyna* contained higher amounts than *S. nigra* and this was true for both the NO_3^- and Zn wash data.

According to the literature, rough leaf surfaces are able to trap more particles than smooth surfaces due to the large frictional drag of such surfaces on airflow (Barthlott and Neinhuis, 1997; Neinhuis and Barthlott, 1998). Surface roughness is often attributed to microstructural ornamentation such as hairs, cuticular folds and epicuticular waxes. Scanning electron microscopy was used to investigate the surface characteristics of these two species. While both species exhibited a certain degree of microstructural ornamentation with respect to cuticular folds, epicuticular wax tubules were observed only on the surface of *C. monogyna. S. nigra* surfaces contained no structures that resembled epicuticular wax crystals. Differences in particulate trapping between these two species may therefore be the result of increased wax ornamentation on the leaves of *C. monogyna.* However,

while an increase in wax ornamentation can increase particulate trapping, this is compensated for by a more effective self cleaning mechanism in rainfall (Barthlott and Neinhuis, 1997; Neinhuis and Barthlott, 1998). Indeed the seasonal results showed no accumulation over the season. Surface amounts of Zn were highest at the start of the season, corresponding to a period of dry weather. It is proposed that the low amounts of surface Zn throughout the remainder of the season was due to the cleansing of leaves by rainfall. The close relationship between rainfall and amounts of surface Zn may be one of the reasons why no significant differences were detected between surface Zn amounts at the two sites since frequent rainfall events did not permit the accumulation of Zn on trees at the motorway.

7.4.1. Achievement of objective

The third objective of the work was to investigate at the deposition of Zn and NO_3^- particles to leaf surfaces as techniques for monitoring traffic exposure. This objective was also achieved. It is proposed that neither measurements of surface NO_3^- nor surface Zn are useful techniques for monitoring traffic exposure as this study found no significant differences between these at the two exposure sites. The lack of differences between the sites may be due to frequent rainfall events which cleansed leaves and thereby prevented the accumulation of particulates.

7.5. Future work and Conclusions

Throughout the much work, the differences in the N assimilation strategies of the two species *S. nigra* and *C. monogyna* have been confirmed. *S. nigra* is characterised by high levels of total leaf N, tissue NO_3^-, xylem NO_3^-, foliar NR activity as well as the ability to use the additional N provided to increase shoot extension dramatically by 93.8%. Such tendencies are characteristic of nitrophilous pioneers as described in Section 1.4.1. *C. monogyna* on the other hand exhibits far less nitrophilous behaviour. The ability of *S. nigra* to utilise the supplementary traffic-derived N may afford this species with a tolerance not only to N pollution but also to other forms of pollution. According to Pearson and Soares (1995) high foliar NR activities afford trees not only with the capacity to buffer against N inputs but also to buffer against any acidic inputs since NO_3^- reduction generates OH^-. According to a recent review of this species (Atkinson and Atkinson, 2002) *S. nigra* has been reported to be tolerant of a range of pollutants including severe pollution from a phosphate fertilizer factory, elevated soil fluoride, sodium, lead and copper as well as atmospheric ozone and sulphur dioxide. In fact *S. nigra* was reported to have the second lowest injury rate when 31 species of trees and shrubs were examined for sulphur dioxide tolerance (Rachwal, 1983).

Species such as *S. nigra* which are not only pollution tolerant but have a high capacity for NO_x uptake and assimilation may be suitable species for planting around NO_x point sources. Not only might these trees serve to ameliorate the amount of local NO_x but they may also reduce the amount of harmful tropospheric

O_3 over wider areas as O_3 is formed from the reactions of NO_x and O_2 in sunlight (Welburn, 1998).

Similarly, according to Beckett *et al.* (1998) levels of harmful atmospheric particulates may be improved by the planting of trees in urban environments. However these authors make no mention of the effect of rainfall on accumulation of surface particulates. Although this study revealed that surface particulates are easily removed from leaf surfaces by rainfall events, trees may still offer potential for cleansing the atmosphere by trapping particulates from the air and channelling them to the soil during rainfall. Indeed the ability of trees to ameliorate air quality has been demonstrated in Chicago where trees are reported to have removed approximately 234 tons of PM_{10} in 1991 resulting in an improvement of air quality has improved hourly air quality by 0.4% (McPherson *et al.*, 1994). Similarly, Nowak *et al.*, 1997 estimated that trees improved air quality was improved by 0.72% in the city o Philadelphia. Trees could also be planted in suburban areas in order to reduce background concentrations of particulates (Beckett *et al.*, 1998).

Part of this work (Chapter 6) looked at the surface Zn amounts of five species in central London, including the pollution tolerant London Plane, *Platanus acerifoli.* Of all the species examined, the trapping of surface Zn was highest on the leaves of *C. monogyna* indicating that this species may be particularly useful at reducing particulate levels. *C. monogyna* is already commonly found in roadside hedgerows where it may be playing an important role in reducing the particulate effects of traffic. This work did not examine the surface amounts of Zn on

coniferous species. These species may have the advantage of offering year round protection but are generally considered less effective at trapping particulates than Broadleaved species since these renew their 'trapping' apparatus i.e. their leaves on a regular basis (Beckett *et al.*, 1998). Similarly for NO_x Hanson *et al.* (1989) reported that deposition of NO_2 to broadleaved species was greater than to conifers.

This work has identified that while *S. nigra* may be a useful species for removing NO_x from the atmosphere, *C. monogyna* may have potential for accumulating particulates and channelling them to the soil during rainfall. Before species are selected for cleansing the atmosphere and planting around point sources it is important to determine their suitability for the habitat. For example, although *S. nigra* may be useful at reducing NO_x levels it may not be suitable for planting along city pavements which can be a hostile environment since they are hot during the day and cold at night. Another consideration if particulates are being transferred to the soil by rainfall is that over time, soil toxicity may occur which could cause physiological damage to the root system. Therefore, it may be useful to investigate resistance to soil toxicity as well as particulate trapping capacity when selecting species for cleansing the atmosphere of particulates.

The work presented in this thesis also allows conclusions to be drawn regarding the suitability of several biomarkers for N deposition and traffic flow. It seems that a major effect of the motorway may have been elevated growth as the result of NO_x inputs from the passing traffic. Simple growth measurements themselves may therefore be used as indicators N deposition. However Speicker (1999)

comments that by themselves growth measurements are not a significantly powerful tool for the diagnosis of specific causes since growth can be stimulated by a number of external factors such as CO_2 supply, light levels and temperature as well as N availability. Other biomarkers for N deposition that have been shown to have potential include $\delta^{15}N$ signatures and NR activity.

$\delta^{15}N$ signatures were shown to be less negative in leaf material collected close to the motorway traffic. The effects of traffic $\delta^{15}N$ signatures is not necessarily dependent on atmospheric uptake of N since the effect was demonstrated in *C. monogyna* and *Quercus* spp., two species for which foliar uptake of atmospheric pollutants is likely to be negligible. Species that are able to take up atmospheric N directly from the atmosphere, such as *S. nigra* may exhibit an even greater response in terms of foliar $\delta^{15}N$ signature since the signature will not be affected by soil and uptake fractionation processes. Unfortunately the foliar signature of *S. nigra* was not investigated as part of this research as there were insufficient individuals along the transect however it may be useful for further research to focus on the $\delta^{15}N$ signatures of trees known to take up N directly from the atmosphere.

NR activity may provide a biomonitoring resource but only if careful consideration is given to the species tested. This work examined the NR activity of 4 species and only significantly higher activities at the motorway site for one of these, *S. nigra,* could be measured. If NR activity is to be used as a biomarker, care also needs to be taken as to the time of year since not only do activities vary

dramatically throughout the growing season but for *S. nigra*, differences between the two sites were only apparent in the summer from late June onwards (See Chapter 5).

Total N per unit of leaf material and tissue NO_3^- did not appear to offer much potential as biomarkers. This is likely to be because traffic-derived N was directed towards new growth which effectively meant that plant N per unit of leaf material remained at a constant level despite the total amount of N within the whole plant increasing. This diluting effect may have also effectively diluted NR activity and may explain why differences in NR were not always significant. It is likely that the $\delta^{15}N$ signature would be diluted in a similar way however despite this problem the effect of the traffic $\delta^{15}N$ signature on the plant $\delta^{15}N$ signature was still detectable.

One disadvantage of this sort of study is that sampling only took place at one site and therefore may not be directly applicable to other habitats. For example whereas this study showed no significant effects of traffic exposure on total N of *C. monogyna* a previous study did detect significant increases with greater deposition levels (Port and Thompson, 1980). It seems that a key factor in the response of vegetation may be the extent to which N deposition is able to stimulate growth. Where soil nutrients such as P are not limiting, biomarkers such as concentrations of total N, total P, tissue NO_3^- and NR may not be useful since N deposition will tend to stimulate growth, allowing concentrations to be held at constant levels. However if N deposition is unable to stimulate additional

growth, for instance because the potential for growth has been satisfied either because the habitat is N saturated or because supply of another nutrient is limiting growth, this is when changes in biomarkers such as total N may occur.

This thesis has demonstrated that although exposed to additional atmospheric N deposition from motorway traffic, trees at Mardley Heath appear to be experiencing few negative effects and are in fact growing vigorously most likely as the result of the fertilizing effect of the traffic-derived N. Two tree species, *S. nigra* and *C. monogyna*, have been identified which may have potential for improving atmospheric concentrations of two different forms of pollution namely NO_x and particulates respectively. It is interesting to note that the planting of trees for the improvement of atmospheric pollution was first recommended over 300 years ago by a British author and founding member of the British Royal Society, John Evelyn (1661). However, relatively little effort has been made to research useful species and planting strategies. The results presented in this thesis suggest that this is definitely an area of research that would benefit from further investigation considering the potential advantages to those living in urban environments in terms of health and general standard of living.

REFERENCES

Achermann, B. and Bobbink, R. (eds.) 2003 Empirical critical loads for nitrogen. Proceedings of an expert workshop, Berne 11-13 November 2002. Environmental Documentation No. 164. Swiss Agency for the Environment. Forests and Landscape SAEFL.

Aerts, R. and Bobbink, R. (1999) The impact of atmospheric nitrogen deposition on vegetation processes in terrestrial, non-forest ecosystems. In: S.J. Langan (ed), The Impact of Nitrogen Deposition on Natural and semi-natural ecosystems, pp 85- 122. Kluwere academic publishers, Dordecht.

Air Quality Expert Group (2004) Nitrogen dioxide in the United Kingdom. DEFRA.

Allen, S.E., Grimshaw, H.E., Parkinson and Quarmby, C. (1974) Nitrogen; Organic constituents – nitrogenous compounds. In *Chemical Analysis of Ecological Materials 1974* pp. 184-206; 265-279. John Wiley, New York.

Allen, A.G., Nemitz, E., Shi, J.P., Harrison, R.M. and Greenwood, J.C. (2001) Size distributions of trace metals in atmospheric aerosols in the United Kingdom. *Atmospheric Environment*, **35**, 4581-4591.

Ammann, M., Siegwolf, R., Pichlmayer, F., Suter, M. (1999) Estimating the uptake of traffic-derived NO_2 from 15N abundance in Norway spruce needles. *Oecologia* **118**, 124-131.

An, B. and Roel, M. (1993) An improved colormetric method to quantify sugar content of plant tissue. *Journal of Experimental Botany,* **44**, 1627-1629.

Andrews, M. (1986) The partitioning of nitrate assimilation between root and shoot of higher plants. *Plant cell Environment*, **9**, 511-519.

APEG (1999). Source Apportionment of Airborne Particulate Matter in the United Kingdom Report of the Airborne Particles Expert Group. ISBN 0-7058-1771-7.

Asner, G.P., Seastedt, T.R. and Townsend, A.R. (1997) The decoupling of terrestrial carbon and nitrogen cycles. *Bioscience*, **47**, 226-234.

Athar, R. and Ahmad, M. (2002) Heavy metal toxicity: effect on plant growth and metal uptake by wheat, and on free living *Azotobacter*. *Water, Air and Soil Pollution*, **138**, 165-180.

Atkinson, M. D. and Atkinson, E. (2002) Sambucus nigra L. *Journal of Ecology*, **90**, 895-923.

Baddeley, J.A., Thompson, D.B.A. and Lee, J.A. (1994) Regional and historical variation in the nitrogen content of *Racomitrium lanuginosum* in Britain in relation to atmospheric nitrogen deposition. *Environmental Pollution*, **84**, 189-196.

Baker, S.J. (1997) Trace and major elements in the atmosphere at rural locations: summary of data obtained for the period 1992-1996. *DETR Report AEAT-2397/20174003*. AEA Technology PLC, Abingdon, Oxford.

Barrie, A. and Lemley, M. (1989) Automated $^{15}N/^{13}C$ analysis of biological material. *International Laboratory*, **19**, 82-91.

Barthlott, W. (1990) Scanning electron microscopy of the epidermal surface in plants. In: Claugher D, ed. Scanning Electron Microscopy in Taxonomy anfd Functional Morphology. Oxford: Clarendon Press, 69-94.

Barthlott, W. and Neinhuis, C. (1997) Purity of the sacred lotus or escape from contamination in biological surfaces. *Planta*, **2002**, 1-8.

Beckett, K.P., Freer-Smith, P.H. and Taylor, G. (1998) Urban woodlands: their role in reducing the effects of particulate pollution. *Environmental Pollution*, **99**, 347-369.

Beevers, L. and Hageman, R.H. (1983) Uptake and reduction of nitrate: Bacteria and higher plants. In: *Encyclopedia of plant physiology, new series vol. 15A* (ed. A. Läuchli and R.L. Bieleski), pp 351-375. Springer-Verlag, Berlin.

Bell, J.N.B. and Treshow, M. (2002) Historical Perspectives. In: *Air Pollution and Plant Life (2nd Ed.)* (ed. J.N.B. Bell and M. Treshow), pp 5-21. John Wiley and Sons, Sussex.

Ben-Zioni, A., Vaadia, Y. and Lips, H. (1971) Nitrate uptake by roots as regulated by nitrate reduction products of the shoot. *Physiologia Plantarum*, **24**, 288-290.

Berg, T., Royset, O. and Steinnes, E. (1995) Moss (*Hylocomium splendens*) used as biomonitor of atmospheric trace element deposition: estimation of uptake efficiencies. *Atmospheric Environment*, **29**, 353-360.

Berg, T., Royset, O., Steinnes, E. and Vadset, M. (1995) Atmospheric trace element deposition: principal component analysis of ICP-MS data from moss samples. *Environmental Pollution*, **88**, 67-77.

Bobbink, R., Hornung, M. and Roelof, J.G.M. (1996) Empirical nitrogen critical loads for natural and semi-natural ecosystems. In: *Manual of methodologies and criteria for mapping critical levels/ loads and geographical areas where they are exceeded. UN ECE Convention on long-range transboundary air pollution, appendix III. 1996* (Eds. Werner, B., Spranger, T.), pp. 1-54 Federal Environmental Agency, Berlin.

Bobbink, R. and Lamers, L.P.M. (2002) Effects of increased nitrogen deposition. In: *Air Pollution and Plant Life,* 2nd edition, (ed. J.N.B. Bell and M. Treshow), pp 201-235. John Wiley & Sons Ltd, Sussex.

Bowsher, C.G., Long, D.M., Oaks, A. and Rothstein, S.J. (1991) Effect of light/dark cycles on expression of nitrate assimilatory genes in maize shoots and roots. *Plant Physiology*, **95**, 281-285.

Brooks, R.R. (1998) Geobotany and hyperaccumulators. In: *Plants that hyperaccumulate heavy metals* (ed. R.R. Brooks), pp 55-94. CAB International, Wallingford, UK.

Brune, A., Urbach, W. and Dietz, K.J. (1995) Differential toxicity of heavy metals is partly related to a loss of preferential extraplasmic compartimentation: a comparison of Cd, Mo, Ni and Zn series. *New Phytologist*, **129**, 403-409.

Buchanan-Wollaston, V. (1997) The molecular biology of leaf senescence. *Journal of Experimental Botany*, **48**, 181-199.

Bytnerowicz, A., Dawson, P.J., Morrison, C.L. and Poe, M.P. (1991) Deposition of atmospheric ions to pine branches and surrogate surfaces in the vicinity of Emerald Lake watershed, Sequoia national park. *Atmospheric Environment*, **25**, 2203-2210.

Calanni, J., Berg, M., Wood, M., Mangis, D., Boyce, R., Weathers, W. and Sievering, H. (1999) Atmospheric nitrogen deposition at a conifer forest: response of tree amino acids in Engelmann spruce needles. *Environmental Pollution,* **105**, 79-89.

Cambell, W.H. and Kinghorn, J.R. (1990) Functional domains of assimilatory nitrate reductases and nitrite reductases. *Trends in Biochemical Science,* **15**, 315-319.

Cambell, W.H. (1996) Nitrate Reductase Biochemistry Comes of Age. *Plant Physiology,* **111,** 355-361.

Carreira, J.A., Harrison, A. F., Sheppard, L.J. and Woods, C. (1997) Reduced soil P availibilty in a Sitka spruce (Picea sitchensis (Bong) Carr) plantation induced by applied acid-mist: Significance in forest decline. *Forest ecology and management*, **92**, 153-166.

Champigny, M.L. and Foyer C. (1992) Nitrate activation of cytosolic protein-kinases diverts photosynthetic carbon from sucrose to amino-acid biosynthesis – Basis for a new concept. *Plant Physiology,* **100,** 7-12.

Chapin, F.S., Van Cleve, K., Tyron, P.R. (1986) Relationship of ion absorption to growth rate in taiga trees. *Oecologia,* **69**, 238-242.

Chapin, F.S., Moilanen, L. and Kielland, K. (1993) Preferential use of organic nitrogen for growth by a non-mychorrhizal arctic sedge. *Nature,* **361,** 150-153.

Chester, R., Bradshaw, G.F., Ottley, C.J., Harrison, R.M., Merrett, J.L., preston, M.R., Rendell, A.R., Kane, M.M. and Jickells, T.D. (1993) The atmospheric distributions of trace metals, trace organics and nitrogen species over the North Sea. *Philosophical Transactions of the Royal Society of London Series A*, **343**, 543-556.

Chiwa, M., Oshiro, N., Miyake, T., Nakatani, N., Kimura, N., Yuhara, T., Hashimoto, N. and Sakugawa, H. (2003) Dry deposition washoff and dew on the surfaces of pine foliage on the urban- and mountain-facing sides of Mt. Gokurakuji, western Japan. *Atmospheric Environment*, **37**, 327-337.

Clement, C.R., Hopper, M.J. and Jones, L.H.P. (1978) The uptake of nitrate by Lolium perenne from flowing nutrient solution I. Effect of concentration. *Journal of Experimental Botany,*. **29**, 453-464.

Clough, E.C.M., Pearson, J. and Stewart, G.R. (1989) Nitrate utilization and nitrogen status in English woodland communities. *Ann. Sci. For.*, **46**, 669-672.

Clough, E.C.M. (1993) *Ecological aspects of nitrate utilization in woody plants.* PhD thesis, University of London, London.

Collier, M.D., Sheppard, L.J., Crossley, A. and Hanke, D.E. (2003) Needle cytokinin content as a sensitive bioindicator of N pollution in Sitka spruce. *Plant, Cell and Environment*, **26**, 1929-1939.

Cotelle, V., Meek, S.E.M, Provan, F., Milne, F.C., Morrice, N. and MacKintosh, C. (2000) 14-3-3s regulate global cleavage of their diverse binding partners in sugar-starved *Arabidopsis* cells. *EMBO Journal*, **12**, 2869-2876.

Cox, R.M. and Malcolm, J.W. (1999) Passive ozone monitoring for forest health assessment. *Water, Air, Soil Pollution*, **116**, 339-344.

De Filippis, L.F. (1981) The effect of heavy metal compounds on the permeability of Chorella cells. Z. *Pflanzenphysiol*, **92**, 39-49.

De Groot, C.C., Marcelis, L.F.M., van den Boogaard, R., Kaiser, M. and Lambers, H. (2003) Interaction of nitrogen and phosphorus nutrition in determining growth. *Plant and Soil*, **248**, 257-268.

Dennis, D.T., Turpin, D.H., Lefebvre, D.D. and Layzell, D.B. (1998) *Plant Metabolism,* Addison Wesley Longman Ltd, Essex. ISBN 0-582-25906-1.

DETR (1999), Air Quality Strategy for England, Scotland, Wales and Northern Ireland, The Stationary Office, Norwich

Diekmann, M. and Falkengren-Grerup, U. (2002) Prediction of species response to atmospheric nitrogen deposition by means of ecological measures and life history traits. *Journal of Ecology,* **90,** 108-120.

Dmuchowski, W. and Bytnerowicz, A. (1995) Monitoring environmental pollution in Poland by chemical analysis of scots pine (*Pinus sylvestris L.*) needles. *Environmental Pollution,* **87,** 87-104.

Draaijers, G.P.J., Ivens, W.P.M.F, and Bleuten, W. (1988) Atmospheric deposition in forest edges measured by monitoring canopy throughfall. *Water, Air, Soil Pollution,* **42,** 129-136.

Drake, B.G., Muehe, M.S., Peresta, G., Gonzalez-Meler, M.A. and Matamala, R. (1996) Canopy photosynthesis of crops and native plant communities exposed to long-term elevated CO_2. *Plant and Soil,* **187**, 111-118.

Elkiey, T. and Ormond, D.P. (1981) Sulphate, total sulphur and total nitrogen accumulation by Petunia leaves exposed to ozone, sulphur dioxide and nitrogen dioxide. *Environmental Pollution,* **24**, 233-241.

Eller, B.M. (1977) Die bedeutung der wachsausblühungen auf blättern von *Kalanchoe pumila* Baker für die absorption der globalstrahlung. *Flora,* **166**, 461-474.

Ericsson, A., Nordén, L-G., Näsholm, T. and Walheim, M. (1993) Mineral nutrient imbalances and arginine concentrations in needles of *Picea abies* (L.) Karst. from two areas with different levels of airborne deposition. *Trees,* **8**, 67-74.

Evelyn, J. (1661) Fumifugium or the inconvenience of the aer and smoak of London dissipated together with some remedies.

Falkengren-Grerup, U., Brunet, J. and Diekmann, M. (1998) Nitrogen mineralisation in deciduous forest soils in south Sweden in gradients of soil acidity and deposition. *Environmental Pollution,* **102**, 415-420.

Falkengren-Grerup, U. and Diekmann, M. (2003) Use of a gradient N-deposition to calculate effect-related soil and vegetation measures in deciduous forests. *Forest Ecology and Management,* **180**, 113-124.

Falkengren-Grerup, U. and Schottelndreier, M. (2004) Vascular plants as indicators of nitrogen enrichment in soils. *Plant Ecology,* **172**, 51-62.

Farmer, A. (2002) Effects of particulates. In: *Air Pollution and Plant Life (2nd Ed.)* (ed. J.N.B. Bell and M. Treshow), pp 187-199. John Wiley and Sons, Sussex.

Fatoki, O.S. (1996) Trace Zinc and Copper concentration in roadside surface soils and vegetation – measurement of local atmospheric pollution in Alice, South Africa. *Environment International,* **22,** 759-762.

Fenn, M.E., Poth, M.A. and Johnson, D.W. (1996) Evidence for nitrogen saturation in the San Bernardino Mountains in southern California. *Forest Ecology and Management*, **82**, 211-230.

Field, C. and Mooney, H.A. (1986) The photosynthesis-nitrogen relationship in wild plants. In: *On the economy of plants form and function* (ed. T.J.Ginish), pp 22-25. Cambridge University Press, Cambridge.

Finzi, A.C., DeLucia, E.H., Hamilton, J.G., Richter, D.D. and Schlesinger, W.H. (2002) The nitrogen budget of a pine forest under free air CO_2 enrichment. *Oecologia,* **132**, 567-578.

Fisk, M.C and Schmidt, S.K. (1996) Microbial responses to nitrogen additions in alpine tundra soil. *Soil Biology and Biochemistry,* **28**, 751-755.

Fitter, A.H. and Hay, R.K.M. (1991) *Environmental physiology of plants.* Academic Press Ltd, London. ISBN 0-12-257764-7.

Forde, B.G. and Clarkson, D.T. (1999) Nitrate and ammonium nutrition of plants: Physiological and molecular perspectives. *Advances in Botanical Research Incorporating Advances in Plant Pathology*, **30**, 1-90.

Fowler, D. (2002) Pollutant deposition and uptake by vegetation. In: *Air Pollution and Plant Life (2nd Ed.)* (ed. J.N.B. Bell and M. Treshow), pp 43-67. John Wiley and Sons, Sussex.

Galloway, J.N. (1998) The global nitrogen cycle: changes and consequences. *Environmental Pollution,* **102,** 15-24.

Gan, S., and Amasion, R.M. (1997) Making sense of senescence. *Plant Physiology*, **113**, 313-319.

Garten, C.T. (1992) Nitrogen isotope composition of ammonium and nitrate in bulk precipitation and forest throughfall. *International Journal of Environmental and Analytical Chemistry*, **47**, 33-45.

Gebauer, G., Melzer, A. and Rehder, H. (1984) Nitrate and nitrate reductase activity in *Rumex obtusifolius* L. I. Differences in organs and diurnal changes. *Oecologia* **63**, 136-142.

Gebauer, G. and Schulze, E.D. (1991) Carbon and nitrogen isotope ratios in different compartments of a healthy and a declining picea-abies forest in the Fichtelgebirge, ne Bavaria. *Oecologia,* **87**, 198-207.

Gessler, A., Schultze, M., Schrempp, S. and Rennenberg, H. (1998a) Interaction of phloem-translocated amino compounds with nitrate net uptake by the roots of beech (*Fagus sylvatica*) seedlings. *Journal of Experimental Botany*, **49**, 1529-1537.

Gessler, A., Schneider, S., Von Sengbusch, D., Weber, P., Hanemann, U., Huber, C. and Rothe, A. (1998b) Field and laboratory experiments on net uptake

of nitrate and ammonium by the roots of spruce (*Picea abies*) and beech (*Fagus sylvatica*) trees. *New Phytologist*, **138**, 275-285.

Golterman, H.L. (1978) *Methods for chemical analysis of fresh waters.* Blackwell Scientific, Oxford.

Gonzales-Real, M.M. and Bailler, A. (2000) Changes in leaf photosynthetic parameters with leaf position and nitrogen content within a rose plant canopy (Rosa hybrida) *Plant Cell and Environment*, **23**, 351–357.

Goulding, K.W.T., Bailey, N.J., Bradbury, N.J., Hargreaves, P., Howe, M., Muphy, D.V., Poulton, P.R. and Willison, T.W. (1998) Nitrogen deposition and its contribution to nitrogen cycling and associated soil processes. *New Phytologist*, **139**, 49-58.

Gniazdowska, A., Mikulska, M. and Rychter, A.M. (1998) Growth, nitrate uptake and respiration rate in bean roots under phosphate deficiency. *Biologia Plantarum*, **41**, 217-226.

Granstedt, R.C. and Huffaker, R.C. (1982) Identification of the leaf vacuole as a major nitrate storage pool. *Plant Physiology*, **70**, 410-413.

Grime, J.P., Hodgson, J.G. and Hunt, R. (1988) *Comparative plant ecology.* Unwin Hyman Ltd, London. ISBN 0-04-445685-9.

Grulke, N.E., Andersen, C.P. and Hogsett, W.E. (2001) Seasonal changes in above and belowground carbohydrate concentrations of ponderosa pine along a pollution gradient. *Tree physiology*, **21**, 173-181.

Handley, L.L., Scrimgeour, C.M. and Raven, J.A. (1998) Stable isotope studies of soil nitrogen. In: *Stable Isotopes* (ed. H. Griffiths), pp 75-88. BIOS Scientific Publishers, Oxford.

Hanson, P.J. and Lindberg, S.E. (1991) Dry deposition of reactive nitrogen compounds: a review of leaf, canopy and non-foliar measurements. *Atmospheric Environment*, **25**, 1615-1634.

Hanson, P.J., Rott, K., Taylor, G.E. Jnr, Gunderson, C.A., Lindberg, S.E. and Ross-Todd, B.M. (1989) NO_2 deposition to elements representative of a forest landscape. *Atmospheric Environment*, **23**, 1783-1794.

Harrison, A.F. (1978) Phosphorus cycles of forest and upland grassland ecosystems and some effects of land management practices. In: *Phosphorus in the environment: Its chemistry and biochemistry* (ed. R. Porter and D.W. Fitzsimons), pp 175-199. Elsevier, Oxford.

Harrison, A.F., Howard, D.M. and Lawson, G.J. (1988) UK soils: their phosphorus sorption capacity and potential for P removal from sewage effluents in emergent hydrophyte treatment systems. *Report to the water research centre*, Medmenham, UK.

Harrison, R.M., Tilling, R. Callén Romero, M. S., Harrad, S. and Jarvis, K. (2003) A study of trace metals and polycyclic aromatic hydrocarbons in the roadside environment. *Atmospheric Environment*, 37, 2391-2402.

Haynes, R.J. and Goh, K.M. (1978) Ammonium and nitrate nutrition of plants. *Biological Reviews*, 53, 465-510.

Heaton, T.H.E., Spiro, B., Madeline, S. and Robertson, C. (1997) Potential canopy influences on the isotopic composition of nitrogen and sulphur in atmospheric deposition. *Oecologia,* **109**, 600-607.

Högberg, P., Högberg, L. and Schinkel, H. (1998) Nitrogen-related root variables of trees along an N-deposition gradient in Europe *Tree Physiology,* **18**, 823-828.

Hörtensteiner, S. and Feller, U. (2002) Nitrogen metabolism and remobilization during senescence. *Journal of Experimental Botany*, **53**, 927-937.

Hose, G.C., James, J.M. and Gray, M.R. (2002) Spider webs as environmental indicators. *Environmental Pollution*, **102,** 725-733.

Hufton, C.A., Besford, R.T. and Wellburn, A.R. (1996) Effects of NO (and NO_2) pollution on growth, nitrate reductase activities and associated protein contents in glasshouse lettuce grown hydroponically in winter with CO_2 enrichment. *New phytologist*, **133**, 495-501.

Huhn, G. and Schulz, H. (1996) Contents of free amino acids in Scots pine needles from field sites with different levels of nitrogen deposition. *New Phytologist*, **134**, 95-101.

Hur, J-S. and Wellburn, A.R. (1994) Effects of atmospheric NO_2 on Azolla-Anabaena symbiosis. *Annals of Botany*, **73**, 137-141.

Husted, S. and Schjoerring, J.K. (995) Apoplastic pH and ammonia concentration in leaves of *Brassica napus* L. *Plant Physiology*, **109,** 1453-1460.

Jacobson, J.S., Lassoie, J.P., Osmeloski, J.F. and Yamada, K. (1989) Changes in foliar elements in red spruce seedlings after exposure to sulphuric acid and nitric acid mist. *Water, Air and Soil pollution*, **48**, 141-159.

Jenkinson, D.S. (1971) The accumulation of organic matter in soil left uncultivated. *Rothamsted Report for 1970, Part 2* 113-137.

Jetter, R., Riederer, M. and Lendzian K.J. (1996) The effects of dry O-3, SO2 and NO" on reconstituted epicuticular wax tubes. *New Phytologist*, **133**, 207-216.

Jung, K., Gebauer, G., Gehre, M., Hofmann, D., Weißflog, L. and Schüürmann, G. (1997) Anthropogenic impacts on natural nitrogen isotope

variations in Pinus sylvestris stands in an industrially polluted area. *Environmental Pollution*, **97**, 175-181.

Kaiser, W.M. and Huber, S.C. (1994) Posttranslational regulation of nitrate reductase in higher plants. *Plant Physiology*, **106**, 817-821.

Kaiser, W.M., Weiner, H. and Huber, S.C. (1999) Nitrate reductase in higher plants: a case study for transduction of environmental stimuli into control of catalytic activity. *Physiolgia Plantarum*, **105**, 385-390.

Kaiser, W.M. and Huber, S.C. (2001) Post-translational regulation of nitrate reductase: mechanism, physiological relevance and environmental triggers *Journal of Botany*, **52**, 1981-1989.

Kauppi, P.E., Mielikainen, K. and Kuusela, K. (1992) Biomass and carbon budget of European forests. *Science*, **256**, 70-74.

Keltjens, W.G. and Van Loenen E. (1989) Effects of Aluminium and mineral-nutrition on growth and chemical-composition of hydroponically grown seedlings of 5 different forest tree species, **119**, 39-50.

Kenis, J.D., Silvente, S.T., Luna, C.M. and Campbell, W.H. (1992) Induction of nitrate Reductase in detached corn leaves: the effect of the age of the leaves. *Physiologia Plantarum* **85**, 49-56.

Killingbeck, K.T. (1996) Nutrients in senescing leaves: Keys to the search for potential resorption and resorption proficiency *Ecology*, **77**, 1716-1727

Kirkham, F.W. (2001) Nitrogen uptake and nutrient limitation in six moorland species in relation to atmospheric nitrogen deposition in England and Wales. *Journal of Ecology*, **89**, 1041-1053.

Kneer, R. and Zenk, M.H. (1992) Phytochelatins protect plant enzymes from heavy metal poisoning. *Phytochemistr.* **31(8),** 2663-2667.

Knops, J.M.H., Bradley, K.L. and Wedin, D.A. (2002) Mechanisms of plant species impacts on ecosystem nitrogen cycling. *Ecology Letters,* **5,** 454-466.

Krywult, M., Hom, J., Bytnerowicz, A. and Percy, K.E. (1994) Deposition of gaseous nitric acid and its effects on foliage of ponderosa pine (Pinus ponderosa L.) *Proceedings of the 16th International Meeting for Specialists in Air Pollution Effects on Forest Ecosystems 'Air Pollution and multiple Stresses'.* Frederichton, Canada.

Kupper, H., Lombi, E., Zhao, F.J. and McCrath, S.P. (2000) Cellular compartmentation of cadmium and zinc in relation to other elements in the hyperaccumulator *Arabidopsis halleri. Planta,* **212,** 75-84.

Lagerwerff, J.T. and Speltch, P.J. (1970) Contamination of roadside soil and vegetation with cadmium, nickel, lead and zinc. *Environ. Sci. Technol.* **4,** 583-586.

Lau, O.W. and Luk, S.F. (2001) Leaves of *Bauhinia blakeana* as indicators of atmospheric pollution in Hong Kong. *Atmospheric Environment,* **35,** 3113-3120.

Lea, P.J., Wolfenden, J. and Wellburn, A.R. (1994) Influence of air pollutants upon nitrogen metabolism. In: *Plant responses to the gaseous environment* (ed. R. Alscher, A.R. Wellburn), pp 279-299. Chapman and Hall, London.

Lea, P.J. (1998) Oxides of nitrogen and ozone: can our plants survive? *New phytologist,* **139,** 25-26.

Lee, J.A., Wooding, S.J. and Press, M.C. (1986) Nitrogen assimilation in an ecological context. In: *Fundamental ecological and agricultural aspects of*

nitrogen metabolism in higher plants. (ed. H. Lambers, J.J. Neeteson and I. Stulen), pp 331-346. editors. Martinus Nijhoff, Dordrecht, The Netherlands.

Lee, J.A. and Caporn, S.J.M. (1998) Ecological effects of atmospheric reactive nitrogen deposition on semi-natural terrestrial ecosystems. *New phytologist,* **139,** 127-134.

L'Hirondelle, S.J., Jacobson, J.S. and Lassoie, J.P. (1992) Acidic mist and nitrogen fertilization effects on growth, nitrate reductase activity, gas exchange, and frost hardiness of red spruce seedlings. *New phytologist,* **121**, 611-622.

Loehle, C. (2003) Competitive displacement in trees in response to environmental change or introduction of exotics. *Environmental Management,* **32**, 106-115.

Luna, C.M., Casano, L.M. and Trippi, V.S. (1997) Nitrate reductase is inhibited in leaves of Trticum aestivum treated with high levels of copper. *Physiologia Plantarum*, **101**, 103-108.

Luna, C.M., Casano, L.M. and Trippi, V.S. (2000) Inhibition of wheat nitrate reductase activity by zinc. *Biologia Plantarum,* **43,** 257-262.

Macnair, M.R., Bert, V., Huitson, S.B. Saumitou-Laprade, P. and Petit, D. (1999) Zinc tolerance and hyperaccumulation are genetically independent characters. *Proc R Soc Lond*, **226**, 2175-2179.

Man, H.M., Abd-El-Baki, G., Stegmann, P., Weiner, H. and Kaiser, W.M. (1999)
The activation state of nitrate reductase is not always correlated with the total nitrate reductase activity in leaves. *Planta*, **209**, 462-468.

Marsh, S. Miller, T., Zhang, X. and Pearson, J. (in press) Tree leaf biomarkers for atmospheric nitrogen deposition. *Water, Air and Soil Pollution*

Marschner, H. (1995) *Mineral nutrition of higher plants.* Academic Press, London. ISBN 0-12-473543-6.

Martinez, C.E. and Motto, H.L. (1999) Solubility of lead, zinc and copper added to mineral soils. *Environmental Pollution,* **107,** 153-158.

Mathys, W. (1975) Enzymes of heavy-metal-resistant and non-resistant populations of *Silene cucubalus* and their interactions with some heavy metals in vitro and in vivo. *Physiologia Plantarum*, **33**, 161-165.

Mathys, W. (1977) The role of malate, oxalate, and mustard oil glucosides in the evolution of zinc resistance in herbage plants. *Physiologia Plantarum,* **40**, 130-136.

McCullough, H. (1967) The determination of ammonia in whole blood by a direct colorimetric method. *Clinica Chimica Acta*, **17**, 297.

McPherson, E.G., Nowak, D.J. and Rowntree, R.E. (1994) Chicago's urban forest ecosystem: results of the Chicago urban forest climate project. *USDA General Technical Report NE-186.*

Mohren, G.M.J., van den Berg, J. and Burger, F.W. (1986) Phosphorus deficiency induced by nitrogen input in Douglas fir in the Netherlands. *Plant Soil,* **95**, 191-200.

Monaci, F., Moni, F., Lanciotti, E., Grechi, D. and Bargagli, R. (2000) Biomonitoring of airborne metals in urban environments: new tracers of vehicle emission, in place of lead. *Environmental Pollution,* **107,** 321-327.

Morgan, S.M., Lee, J.A. and Ashenden, T.W. (1992) Effects of nitrogen oxides on nitrate assimilation in bryophytes. *New Phytologist*, **120**, 89-97.

Muller, B., Touraine, B. (1992) Inhibition of NO_3 uptake by various phloem-translocated amino acids in soybean seedlings. *Journal of Experimental Botany,*. **43**, 617-623.

Nadelhoffer, K.J., Emmett, B.A., Gundersen, P., Kjonaas, O.J., Koopmans, C.J., Schleppi, P., Tietema, A. and Wright, R.F. (1999) Nitrogen deposition makes a minor contribution to carbon sequestration in temperate forests. *Nature,* **398**, 145-148.

Nasholm, T. (1998) Qualitative and quantitative changes in plant nitrogen acquisition induced by anthropogenic nitrogen deposition. *New phytologist,* **139**, 87-90.

Nasholm, T., Ekblad, A., Nordin, A., Giesler, R. Hogberg, M. and Hogberg, P. (1998) Boreal forest plants take up organic nitrogen. *Nature,* **392**, 914-916.

Neff, J.C., Townsend, A.R., Gleixner, G., Lehman, S.J., Turnbull, J. and Bowman, W.D. (2002) Variable effects of nitrogen additions on the stability and turnover of soil carbon. *Nature,* **419**, 915-917.

Neinhuis, C. and Barthlott, W. (1998) Seasonal changes of leaf surface contamination in beech, oak, and ginkgo in relation to leaf micromorphology and wettability. *New phytologist,* **138**, 91-98.

Nilsson, J. (1986) Critical loads for nitrogen and sulphur. Mikjørapport 11, pp 232, Nordic Council of Ministers, Copenhagen.

Nilsson, J. and Grennfelt, P. eds. (1988) Critical loads for sulphur and nitrogen. Report from a workshop held at Skokloster, Sweden, 19-24 March 1988, UN_ECE/Nordic Council of Ministers, Copenhagen, Denmark, *Nord* **15.**

Nishimura, H., Hayamizu, T. and Yanagisawa, Y. (1986) Reduction of NO_2 to NO by rush and other plants. *Environmental Science and Technology,* **20**, 413-416.

Noodén, L.D. and Letham, D.S. (1986) Cytokinin control of monocarpic senescence in soybean. In: *Plant Growth Substances* (ed. M. Bopp), pp 324-332. Springer-Verlag, Berlin.

Norby, R. J., Weerasuriya, Y. and Hanson, P. (1989) Induction of nitrate reductase activity in red spruce needles by NO_2 and HNO_3 vapor. *Canadian Journal of Forest Research*, **19**, 889-896.

Nordin, A. and Nasholm, T. (1997) Nitrogen storage forms in nine boreal understorey plant species. *Oecologia,* **110**, 487-492.

Nriagu, J. (1989) A global assessment of natural sources of atmospheric trace-metals. *Nature,* **338**, 47-49.

Oaks, A., Wallace, W. and Stevens, D. (1972) Synthesis and turnover of nitrate Reductase in corn roots. *Plant Physiology,* **50**, 649-654.

Oaks, A. (1991) Nitrogen assimilation in roots: a re-evaluation. *BioScience*, **42**, 103-111.

Oghogharie, C.G.O. and Pate, J.S. (1972) Exploration of the nitrogen transport system of a nodulated legume using ^{15}N. *Planta,* **104**, 35-49.

Otten, P., Injuk, J. and Van Grieken, R. (1994) Elemental concentrations in atmospheric particulate matter sampled on the North Sea and the English Channel. *Science of the Total Environment,* **155**, 131-149.

Pacyna, J.M. (1986) In: *Toxic metals in the atmosphere* (Ed. J.O. Nriagu and C.I. Davidson). Wiley, New York.

Parsons, R. and Sunley, R.J. (2001) Nitrogen nutrition and the role of root-shoot nitrogen signalling particularly in symbiotic systems. *Journal of Experimental Botany,* **52**, 435-443.

Pearson, J. Clough, E.C.M. and Kershaw, J.L. (1989) Comparative study of nitrogen assimilation in woodland species. *Ann. Sci. For.*, **46**, 663-665.

Pearson, J. and Stewart, G.R. (1993) Tansley Review No. 56 The deposition of atmospheric ammonia and its effects on plants. *New phytologist,* **125**, 283-305.

Pearson, J. and Ji, Y.M. (1994) Seasonal variation of leaf glutamine synthetase isoforms in temperate deciduous trees strongly suggests different functions for the enzymes. *Plant, Cell and Environment,* **17**, 1331-1337.

Pearson, J. and Soares, A. (1995) A hypothesis of plant susceptibility to atmospheric pollution based on intrinsic nitrogen metabolism: why acidity really is the problem. *Water, Air and Soil Pollution*, **85**, 1227-1232.

Pearson, J. and Soares, A. (1998) Physiological responses of plant leaves to atmospheric ammonia and ammonium. *Atmospheric Environment*, 32, 533-538.

Pearson, J., Clough, E.C.M., Woodall, J., Havill, D.C. and Zhang, X-H. (1998) Ammonia emissions to the atmosphere from leaves of wild plants and *Hordeum vulgare* treated with methionine sulphoximine. *New phytologist,* **138**, 37-48.

Pearson, J., Wells, D.M., Seller, K.J., Bennett, A., Soares, A., Woodall, J. and Ingrouille, M.J. (2000) Traffic exposure increases natural ^{15}N and heavy metal concentrations in mosses. *New Phytologist*, **147**, 317-326.

Pedroso, M.C., Magalhaes, J.R. and Durzan, D. (2000) A nitric oxide burst precedes apoptosis in angiosperm and gymnosperm callus cells and foliar tissues. *Journal of Experimental Botany*, **51**, 1027-1036.

Peoples, M.B. and Dalling, M.J. (1988) The inter-play between proteolysis and amino acid metabolism during senescence and nitrogen re-allocation. In: Senescence and aging in plants (ed. L.D Nooden and A.C. Leopold), pp 181-217. Academic Press, New York.

Peterson, P.J. (1969) The distribution of zinc-65 in *Agrostis tenuis* Sibth. And *A. stolonifera* L. tissues. *Journal of Experimental Botany*, **20**, 863-887.

Pitcairn, C.E.R., Fowler, D. and Grace, J. (1995) Deposition of fixed atmospheric nitrogen and foliar nitrogen content of bryophytes and *Calluna vulgaris* (L.) Hull. *Environmental Pollution*, **88**, 193-205.

Pitcairn, C.E.R., Skiba, U.M, Sutton, M.A., Fowler, D., Munro, R. and Kennedy, V. (2002) Defining the spatial impacts of poultry farm ammonia emissions on species composition of adjacent woodland groundflora using Ellenberg Nitrogen Index, nitrous oxide and nitric oxide emissions and foliar nitrogen as marker variables. *Environmental Pollution*, **119**, 9-21.

Pitcairn, C.E.R., Fowler, D., Leith, I.D., Sheppard, L.J., Sutton, M.A., Kennedy, V. and Okello, E. (2003) Bioindicators of enhanced nitrogen deposition. *Environmental Pollution*, **126**, 353-361.

Port, G.R. and Thompson, J.R. (1980) Outbreaks of insect herbivores on plants along motorways in the United Kingdom. *Journal of Applied Ecology*, **17**, 649-656.

QUARG (1993) Urban Air Quality in the United Kingdom, First Report of the Quality of Urban Air Review Group, Department of the Environment. ISBN 0 95207 7116.

Raab, T.K. and Terry, N. (1995) Carbon, nitrogen and nutrient interactions in *Beta vulgaris L.* as influenced by nitrogen source, NO_3^- versus NH_4^+. *Plant Physiology*, **107**, 575-584.

Rachwal, L. (1983) Tolerance variability of trees and shrubs to high concentrations of SO_2 and heavy metals. *Aquilo. Botanica*, **19**, 342-353.

Raven, J.A. (1986) Biochemical disposal of excess H^+ in growing plants? *New phytologist*, **101**, 25-77.

Raven, J.A. (1988) Acquisition of nitrogen by the shoots of land plants: its occurrence and implications for acid-base regulation. *New Phytologist,* **109**, 1-20.

Reuss, J.O. and Johnson, D.W. (1986) *Acidic deposition and the acidification of soils and waters.* Springer, New York. pp 1-119.

Rideout, J.W., Chaillou, S., Raper, C.D. and Morot-Gaudry, J.F. (1994) Ammonium and nitrate uptake by soybean during recovery from nitrogen deprivation. *Journal of Experimental Botany,* **45**, 23-33.

Rogers, H.H., Campbell, J.C. and Volk, R.J. (1979) Nitrogen-15 dioxide uptake and incorporation by *Phaseolus vulgaris* L. *Science,* **206**, 333-337.

Runge, M. (1983) Physiology and ecology of nitrogen nutrition In: *Encyclopedia of Plant Physiology, vol 12C. Physiological Plant Ecology III. 1983* (Eds. Lange, O.L., Nobel, P.S., Osmond, C.B., Ziegler, H.) pp 163-200 Berlin, Heidelberg, New York: Springer-Verlag.

Sabaratnam, S., Gupta, G. and Mulchi, C. (1988) Effects of nitrogen dioxide on leaf chlorophyll and nitrogen content of soybean. *Environmental Pollution,* **51**, 113-120.

Sanders, G.A., Skärby, L., Ashmore, M.R. and Fuhrer, J. (1995) Establishing critical levels for the effects of air pollution on vegetation. *Water, Air and Soil Pollution,* **85,** 189-200.

Sanz, M.J, Carratalá, Gimeno, C. and Millán, M.M. (2002) Atmospheric nitrogen deposition on the east coast of Spain: relevance of dry deposition in semi-arid Miditerranean regions. *Environmental Pollution*, **118**, 259-272.

Scherbatskoy, T. and Tyree, M.T. (1990) Kinetics of exchange of ions between artificial precipitation and maple leaf surfaces. *New Phytologist*, **114**, 703-712.

Schmidt, S. and Stewart, G.R. (1997) Waterlogging and fire impacts on nitrogen availability and utilization in a subtropical wet heathland (*wallum*) *Plant, Cell and Environment*, **20**, 1231-1241.

Schmidt, S., Stewart, G.R., Turnbull, M.H., Erskine, P.D. and Ashwath, N. (1998) Nitrogen relations of natural and disturbed plant communities in tropical Australia. *Oecologia*, **117**, 95-104.

Schulze, E.D. (1989) Air pollution and forest decline in a spruce (*Picea abies*) forest. *Science*, **244**, 776-783.

Siebrecht, S. and Tischner, R. (1999) Changes in the xylem exudates composition of poplar (*Populus tremula* x *P. alba*)-dependent on the nitrogen and potassium supply. *Journal of Experimental Botany*, **50**, 1797-1806.

Singh, S.N. and Rao, D.N. (1981) Certain responses of wheat plants to cement dust pollution. *Environmental Pollution*, **24**, 75-81.

Smarrelli, J., Jr. and Cambell, W.H. (1983) Heavy metal inactivation and chelator stimulation of higher plant nitrate reductase. *Biochimica et Biophysica Acta* **742**, 435-445.

Smirnoff, N., Todd, P. and Stewart, G.R. (1984) The occurrence of nitrate reduction in the leaves of woody plants. *Annals of Botany*, **54**, 363-374.

Smirnoff, N. and Stewart, G.R. (1985) Nitrate assimilation and translocation by higher plants: Comparative physiology and ecological consequences. *Physiol. Plant.*, **64**, 133-140.

Smirnoff, N. and Stewart, G.R. (1987) Nitrogen assimilation and zinc toxicity to zinc-tolerant and non-tolerant clones of *Deschampsia cespitosa* (L.) beauv. *New phytologist*, **107**, 671-680.

Soares, A., Ming, J.Y. and Pearson, J. (1995) Physiological indicators and susceptibility of plants to acidifying atmospheric pollution: a multivariate approach. *Environmental Pollution*, **87**, 159-166.

Solomonson, L.P and Barber, M.J. (1990) Assimilatory nitrate reductase: functional properties and regulation. *Annual Revue of Plant Physiology and Plant Molecular Biology*, **41**, 225-253.

Spangenberg, A. and Kölling, C. (2004) Nitrogen deposition and nitrate leaching at forest edges exposed to high ammonia emissions in southern Bavaria. *Water Air And Soil Pollution*, **152**, 233-255.

Spieker, H. (1999) Overview of recent growth trends in European forests. *Water Air and Soil Pollution*, **116**, 33-46.

Srivastava, H.S. and Ormrod, D.P. (1986) Effects of nitrogen dioxide and nitrate nutrition on nodulation, nitrogenase activity, growth, and nitrogen content of bean plants. *Plant Physiology*, **81**, 737-741.

Srivastava, H.S. and Ormrod, D.P. (1989) Effects of nitrogen dioxide and nitrate nutrition effects on nitrate reductase activity and nitrate content of bean leaves. *Environmental and Experimental Biology*, **29**, 433-438.

Srivastava, H.S., Wolfenden, J., Lea, P.J. and Wellburn, A.R. (1994) Differential responses of growth and nitrate Reductase activity in wild type and

NO_2- tolerant barley mutants to atmospheric NO_2 and nutrient nitrate. *Journal of Plant Physiology,* **143**, 738-743.

Stephens, D.W., Millard, P., Turnbull, M.H. and Whitehead, D. (2001) The influence of nitrogen supply on growth and internal recycling of nitrogen in young *Nothofagus fusca* trees. *Australian Journal of Plant Physiology,* **28**, 249-255.

Stewart, G.R and Orebamjo, T.O. (1979) Some unusual characteristics of nitrate reduction in *Erythrina senegalalensis* D.C. *New Phytologist*, **83**, 311-319.

Stewart, G.R., Joly, C.A. and Smirnoff, N. (1992) Partitioning of inorganic nitrogen assimilation between the roots and shoots of cerrado and forest trees of contrasting plant communities of South East Brazil. *Oecologia,* **91,** 511-517.

Stewart, G.R., Aidar, M.P.M, Joly, C.A. and Schmidt, S. (2002) Impact of point source pollution on nitrogen isotope signatures ($\delta^{15}N$) of vegetation in SE Brazil. *Oecologia*, **131**, 468-472.

Strengbom, J., Olofsson, J., Witzell, J. and Dahlgren, J. (2003) Effects of repeated damage and fertilization on palatability of Vaccinium myrtillus to grey sided voles, Clethrionomys rufocanus. *OIKOS*, **103**, 133-141.

Stulen, I., Perez-Soba, M., De Kok, L.J. and Van der Eerden, L. (1998) Impact of gaseous nitrogen deposition on plant functioning. *New phytologist,* **139**, 61-70.

Sverdrup, H., Warfvinge, P., Blake, L. and Goulding, KW.T. (1995) Modelling recent and historic soil data from the Rothamsted Experimental Station, UK using SAFE. *Agriculture, Ecosystems and Environment,* **53**, 161-177.

Temperton, V.M., Millard, P. and Jarvis, P.G. (2003) Does elevated atmospheric carbon dioxide affect internal nitrogen allocation in the temperate trees *Alnus glutinosa* and *Pinus sylvestris*. *Global Change Biology*, **9**, 286-294.

Tessier, J.T. and Raynal, D.J. (2003) Use of nitrogen to phosphorus ratios in plant tissue as an indicator of nutrient limitation and nitrogen saturation. *Journal of Applied Ecology*, **40**, 523-534.

The Air Quality Strategy (1999) The Air Quality Strategy for England, Scotland, Wales and Northern Ireland. Department of the Environment.

Thimonier, A., Dupouey, J.L., Bost, F and Becker, M. (1994) Simultaneous eutrophication and acidification of a forest ecosystem in North-East France *New phytologist*, **126**, 533-539.

Thoene, B., Schröder, P., Papen, H., Egger, A. and Rennenberg H. (1991) Absorption of atmospheric NO_2 by spruce (Picea abies L. Karst.) trees I. NO_2 influx and its correlation with nitrate reduction. *New Phytologist*, **117**, 575-585.

Thomas, F.M. and Buttner, G. (1998) Correlations between nutrient status and degree of foliation in mature oak stands of northwestern Germany. *Forstwissenschaftliches Centralblatt*, **117**, 115-128.

Thomas, F.M. and Hilker, C. (2000) Nitrate reductase in leaves and roots of young pedunculate oak (*Quercus robur*) growing on different nitrate concentrations. *Environmental and Experimental Botany*, **43**, 19-32.

Touraine, B., Muller, B. and Grignon, C. (1992) Effect of phloem-translocated malate on NO_3^- uptake by roots of intact soybean plants. *Plant Physiology*, **99**, 1118-1123.

Turner, R.G. and Marshall, C. (1972) The accumulation of Zn by subcellular fractions of roots of *Agrostis tenuis* Sibth. In relation to zinc tolerance. *New Phytologist*, **71**, 671-676.

Tyler, G. (1987) Probable effects on soil acidification and nitrogen deposition on the floristic composition of oak (Quercus robur L.) forest. *Flora*, **179**, 165-170.

Van assche, F. and Clijsters, H. (1986) Inhibition of photosynthesis in Phaseolus vulgaris by treatment with toxic concentrations of zinc: effects on electron transport and photo-phosphorylation. *Physiologia Plantarum*, **66**, 717-721.

VanDijk, H.F.G. and Roelofs, J.G.M. (1988) Effects of excessive ammonium deposition on the nutritional-status and condition of pine needles. *Physiologia Plantarum*, **73**, 494-501.

Veen, A.W.L., Klassen, W., Kruijt, B. and Hutjes, R.W.A. (1996) Forest edges and the soil vegetation-atmosphere interaction at the landscape scale: the state of affairs. *Progress Phys. Geogr.*, **20**, 292-310.

Vinton, M.A. and Burke I.C. (1995) Interactions between individual plant species and soil nutrients status in shortgrass steppe. *Ecology*, **76**, 1116-1133.

Vitousek, P.M. and Howarth, R.W. (1991) Nitrogen limitation on land and in the sea: how can it occur? *Biogeochemistry*, **13**, 87-115.

Vitousek, P.M. (1994) Beyound global warming: ecology and global change. *Ecology*, **75**, 1861-1876.

Weathers, K.C., Lovett, G.M., Likens, G.E. and Lathrop, R. (2000) The effect of landscape features on deposition to Hunter Mountain, Catskill Mountains, New York. *Ecological Applications*, 10, 528-540.

Wedin, D.A. and Tilman, D. (1996) Influence of nitrogen loading and species composition on the carbon balance of grasslands. *Science,* **274**, 1720-1723.

Wellburn, A.R. (1990) Atmospheric nitrogenous compounds and ozone – is NO_x fixation by plants a possible solution? *New phytologist,* **139**, 5-9.

Wellburn, A.R. (1994) *Air pollution and climate change.* Longman Scientific and Technical. Essex. ISBN 0-470-20006-5.

Wellburn, A.R. (1998) Atmospheric nitrogenous compounds and ozone – is NO_x fixation by plants a possible solution? *New Phytologist*, **139**, 5-9.

Wingsle, G., Näsholm, T., Lundmark, T. and Ericsson, A. (1987) Induction of nitrate reductase in needles of Scots pine seedlings by NO_x and NO_3^-. *Physiologia Plantarum*, **70**, 399-403.

Wolfenden, J. and Wellburn, A.R. (1986) Cellular readjustment of barley seedlings to stimulated acid rain. *New Phytologist,* **104**, 97-109.

Woolgrove, C.E. and Woodin, S.J. (1996) Current and historical relationships between the tissue nitrogen content of a snowbed bryophyte and nitrogenous air pollution. *Environmental Pollution,* **91**, 283-288

World Health Organisation (WHO) (1999) *Health costs due to road traddic-related air pollution.* WHO, Copenhagen.

Yamasaki, H. and Sakihama, Y. (2000) Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: in vitro evidence for the NR-dependent formation of active nitrogen species. *Febs Letters,* **468**, 89-92.

Ylaranta, T. (1995) Effect of road traffic on heavy-metal concentrations of plants. *Agricultural Science in Finland,* **4**, 35-48.

Zeevart, A.J. (1974) Induction of nitrate reductase by NO_2. *Acta Botanica Neerlandica,* **23**, 345-346.

Zhao, F.J., Hawkesford, M.J. and McGrath, S.P. (1999a) Sulphur assimilation and effects on yield and quality of wheat. *Journal of Ceral Science*, **30**, 1-17.

Zhao, F.J., Salmon, S.E., Withers, P.J.A., Monaghan, J.M., Evans, E.J., Shewry, P.R. and McGrath, S.P. (1999b) Variation in the breadmaking quality and rheological properties of wheat in relation to sulphur nutrition under field conditions. *Journal of Ceral Science*, **30**, 19-31.

Zhao, F.J., Lombi, E., Breedon, T. and McGrath, S.P. (2000) Zinc hyperaccumulation and cellular distribution in Arabidopsis halleri. *Plant Cell and Environment*, **23**, 507-514.

Zhen, R.G, Leigh, R.A. (1990) Nitrate accumulation by wheat (Triticum aestivum) in relation to growth and tissue N concentrations. In: *Plant nutrition physiology and applications* (ed. M.L. van Beusichen), pp 17-20. Kluwer Academic, Norwell, Massachusetts, USA.

Zhen, R.G., Smith, S.J. and Miller, A.J. (1992) A comparison of nitrate-selective microelectrodes made with different nitrate sensors and the measurement of intracellular nitrate activities in cells of excised barley roots. *Journal of Experimental Botany,* **43**, 131-138.

Zielke, H.R. and Filner, P. (1971) Synthesis and turnover of nitrate reductase induce by nitrate in cultured tobacco cells. *Journal of Biological Chemistry* **246**, 1772-1779.

Zinke, P.J. (1980) *Influence of chronic air pollution on mineral cycling in forest. In: Proceedings of the symposium on effects of air pollutants on Mediterranean and temperate forest ecosystems, 22-27 June 1980, Riverside CA.* (ed. P.R. Miller), pp 88-99. For. Serv. Gen. Tech. Rep.